

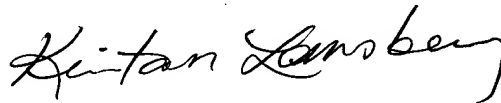
## Remarks

The substitute specification has been amended to insert the cross-reference to related application information, specifying that the present application is a national phase application under 35 U.S.C. § 371 of International Application No. PCT/JP98/01728, filed April 15, 1998, which claims priority to Japanese Patent Application No. 97808/1997, filed April 15, 1997, Japanese Patent Application No. 151434/1997, filed June 9, 1997, Japanese Patent Application No. 217897/1997, filed August 12, 1997, Japanese Patent Application No. 224803/1997, filed August 21, 1997 and Japanese Patent Application No. 332241/1997, filed December 2, 1997.

This amendment does raise new issue for further search and consideration as this information was contained in the declaration as previously submitted in the application. This amendment was not presented earlier because the substitute specification was filed on this same date.

If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application or if the Examiner has any questions regarding this application, the Examiner is invited to contact Applicants' undersigned representative at (202) 942-5186.

Respectfully submitted,



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## DESCRIPTION

### NOVEL PROTEIN AND METHOD

#### AN OCIF-BINDING MOLECULE (OBM), NUCLEIC ACID ENCODING, AND PROCESS FOR PRODUCING THE PROTEIN

##### Field of Technology

##### TECHNICAL FIELD

The present invention relates to a novel protein (OCIF- binding molecule, ~~the protein may be;~~ hereinafter called it may be referred to as "OBM"), which binds to osteoclastogenesis inhibitory factor (~~hereinafter it may be called OCIF~~), and a production method to produce this protein thereof. ~~The~~ In addition, the present invention also relates to DNA ~~encoding which this encodes the protein, proteins containing the a protein having~~ an amino acid sequence encoded by this DNA, a method for genetically producing the preparation of this protein-utilizing genetic engineering techniques, and a pharmaceutical compositions comprising this protein.

The present invention ~~further also~~ relates to a methods for of screening, using this protein and the DNA, substances to control the for: a substance which controls expression of this protein, substances inhibiting a substance which inhibits or regulating modifies the biological activity of this protein, or receptors transducing a receptor which binds the signal of protein and transmits the activity thereof, methods of using the protein by interacting with this protein, to or the DNA; the substances obtained by the screening these methods; and pharmaceutical compositions comprising the obtained substances. In addition, the present invention also relates to an antibody to the protein, a method for the production thereof, a method for measuring the protein with the antibody, and to pharmaceutical compositions which comprise the resulting substances an agent comprising the antibody.

~~The present invention further relates to antibodies against this protein, methods for preparing the antibodies, and pharmaceutical compositions comprising these antibodies.~~

~~Background Art~~H6, 4400, 839, 5

## **BACKGROUND ART**

~~Bone metabolism is dependent~~depends on the overall activity of osteoblasts ~~which responsible control~~for bone formation, and osteoclasts ~~which control~~, responsible ~~for~~for bone resorption. ~~Abnormality of~~It is assumed that bone metabolism ~~abnormality~~is ~~considered to be~~caused by an imbalancedue to loss of the ~~balance~~between bone formation and the bone resorption. ~~Osteoporosis~~As diseases involving bone metabolism ~~abnormality~~, osteoporosis, hypercalcemia, bone Paget's disease, renal osteodystrophy, chronic rheumatoid arthritis, osteoarthritis, rheumatoid arthritis and the like osteoarthritis are known as diseases accompanying abnormality of bone metabolism. ~~Osteoporosis is a~~typical disease caused by such abnormality of A representative of these bone metabolism ~~abnormality diseases is~~osteoporosis. This disease ~~is generated~~occurs when bone resorption by osteoclasts exceeds bone formation by osteoblasts. ~~The disease and~~is characterized by a equal decrease in ~~both the bone calcified~~both ~~substances~~substances and the bone matrix. ~~Although the~~The mechanism for crisis of this disease is not completely elucidated yet fully clarified, while it is a disease with pain in bone and bone fracture due to the increased fragility of bone. Along with an increase in the population of aged people, this disease causes aged people to fracture bone, resulting in bones, makes them fragile, and may result in confinement in bed. This disease is becoming already a social problem because it increases the number of bedridden aged persons as the aged population becomes larger. Development of therapeutic agent, so that medicaments for this treating the disease is are urgently desired. needed to be developed. ~~Disease due to a decrease in bone mass~~It is expected to that osteopenia due to bone metabolism abnormality can be curedtreated by suppressingstimulating bone formation, inhibiting bone resorption, ~~accelerating bone formation~~, or improving the balance between them. That is, ~~bone resorption and formation~~. Bone formation is expected ~~to be~~increasestimulated by accelerating proliferationpromoting the growth,

differentiation, ~~or activation~~ and functions of osteoblasts, which ~~form~~ are responsible for bone formation, ~~or by suppressing proliferation,~~ the differentiation, or activation of osteoclast precursor cells to osteoclasts ~~which resorb~~ and maturation thereof, or suppressing osteoclast function such as bone-resorbing activity. ~~In recent years~~ At present, ~~strong interest has been directed to~~ hormones, substances of low molecular weight substances, or physiologically active proteins exhibiting such activities, ~~and energetic basic research and development is underway on these subjects~~ activity are being studied and developed.

~~Drugs such as~~ As agents for treating bone-relating diseases and shortening treatment periods thereof, a calcitonin-containing ~~agents~~ formulation, the active-form of vitamin D<sub>3</sub> ~~containing agents~~ formulation, hormone ~~agents containing~~ (estradiol, ipriflavone, vitamin K<sub>2</sub>) ~~containing formulation~~ and bisphosphonate ~~compounds have-~~ based compound are already been known as drugs to treat and shorten clinically available. Furthermore, to develop medicaments with less side effects and excellent effectiveness, clinical trials of the treatment period of diseases related to bone. ~~Clinical tests are in progress on active-form of~~ vitamin D<sub>3</sub> derivatives, estradiol derivatives, and bisphosphonate-based compounds of the second and the third generation to develop therapeutic agents with excellent efficacy and minimal side effects have been held.

However, ~~therapiessince~~ such methods for treatment using these agents were found drugs are not necessarily satisfactory sufficient in terms of efficacy effectiveness and therapeutic results. Development of treatment, novel therapeutic agents which medicaments that are safer and with have higher efficacy is urgently desired effectiveness have been expected to be developed. ~~Some agents~~ Moreover, among medicaments used for their treatment of diseases related to bone metabolism diseases, there are those which can be used only limitedly for treating a restricted kind of disease due to their side effects thereof. Furthermore In addition, treatments using two or more agents in combination are currently the mainstream in the treatment of diseases related at present, to treat bone metabolism diseases such as osteoporosis, treatment with combined use of more than one medicament is currently usual. From such a point of view, ~~development of drugs~~ a medicament having different action mechanisms ~~different~~ from



those of the conventional drugs, and exhibiting ones with higher efficacy/effectiveness and minimal/less side effects is desired have been expected to be developed.

As mentioned/described above, the cells controlling/responsible for bone metabolism are osteoblasts and osteoclasts. These cells are It is known to have close mutual interactions called "that these cells closely interact with each other, and this phenomenon is regarded as coupling". Specifically That is, it has been reported that the differentiation and maturation of osteoclasts are stimulated or suppressed by cytokines such as Interleukins, interleukins 1 (IL-1), 3 (IL-3), 6 (IL-6), and 11 (IL-11), granulocyte- macrophage- colony -stimulating factors (GM-CSF), macrophage- colony -stimulating factors (MGM-CSF), Interferon- $\gamma$  gammas (IFN- $\gamma$ ), tumor necrosis factors  $\alpha$  (TNF- $\alpha$ ), and transforming growth factor- $\beta$  factors  $\beta$  (TGF- $\beta$ ), and the like, which are secreted by osteoblastic stroma from osteoblast-like stroma cells are known to accelerate or suppress differentiation or maturation of osteoclasts (Raisz: Disorders of Bone and Mineral Metabolism, 287- to 311, 1992; Suda *et al.*: Principles of Bone Biology, 87- to 102, 1996; Suda *et al.*: Endocrine Reviews, 4, 266- to 270, 1995, 1995, Lacey *et al.*: Endocrinology, 186, 2369- to 2376, 1995). It has been reported is known that osteoblastic/osteoblast-like stromal cells play an important role in the differentiation and maturation of osteoclasts, as well as in and expression of mature osteoclast functions/function, such as bone resorption by mature osteoclasts, through cell-intercellular binding to cell contact with immature osteoclast precursors/precursor cells of osteoclasts or (mature) osteoclasts.—A factor called As a factor involved in osteoclastogenesis by the intercellular binding, a molecule known as osteoclast differentiation factor (ODF<sub>F</sub>) (Suda *et al.*: Endocrine Rev. 13:13, 66- to 80, 1992; Suda *et al.*: Bone 17, 87S- to 91S, 1995) which is thought to be expressed on the membrane of osteoblastic/the osteoblast-like stromal cells and involved in the formation of osteoclasts through cell-to-cell contact/cell is predicted. According to this hypothesis/assumption, an ODF $\alpha$  receptor is present for ODF exists in the osteoclast precursor cells of osteoclasts/cell. However, so far neither the ODF nor and the receptor has been are not yet either purified or identified. There, and there are also no reports relating to on their characteristics, action mechanism/mechanisms or structure/end structures. Thus As just described, the mechanism involved in for differentiation and maturation of osteoclasts has

~~not yet been sufficiently elucidated. Clarification~~fully understood yet, and it is expected  
that full understanding of this mechanism will greatly significantly contribute not only  
to the basic medicine, field of experimental medicines but also to the  
development/developments of novel drugs/agents for the treatment of diseases associated  
with/treating bone metabolism abnormality of bone metabolism, based on the novel action  
mechanism.

~~The~~Under the circumstances, the present inventors have ~~conducted~~made  
~~extensive/intensive~~studies in view of this situation and discovered an/found  
osteoclastogenesis inhibitory factors (OCIF) in ~~a~~the culture ~~broth~~solution of human  
~~embryonic/fetal~~lung fibroblast, fibroblasts IMR-90 (ATCC ~~Deposition No.~~ CCL186)  
(WO 96/26217).

~~The~~Then, the present inventors have ~~been successful/succeeded~~in DNA cloning  
~~DNA encoding of~~OCIF, production of a recombinant OCIF in/using an animal cells, and  
confirmation of *in vivo* ~~pharmaceutical/medicinal~~effects/virtues (bone metabolism  
improving effect on bone metabolism, etc.) of the recombinant OCIF. OCIF is expected  
~~to be used as an agent for a medicament that has higher effectiveness and causes less side~~  
effects than the prevention or treatment of conventional one and can prevent and treat  
diseases related to abnormality of/associated with bone metabolism, with higher efficacy  
than conventional drugs and less side effects abnormality.

#### Disclosure of the Invention**DISCLOSURE OF THE INVENTION**

The present inventors have intensively searched for the existence of a protein  
~~which/that~~binds to osteoclastogenesis inhibitory factor (OCIF) and discovered by using  
OCIF. As a result, the inventors have found that an-OCIF- binding protein is specifically  
expressed on ~~the an~~osteoblastic/osteoblast-like stromal cells cultured in the presence of a  
bone resorption factors such as the active-form of vitamin D<sub>3</sub> and parathyroid hormone  
(PTH). ~~In addition~~Furthermore, the present inventors have investigated as a result of  
studying the characteristics and physiological functions of ~~this~~-OCIF- binding protein ~~and~~  
~~found that, the protein exhibits~~was found to have biological activity ~~of as~~a factor which  
supports or promotes the so-called osteoclast differentiation and maturation ~~from factor,~~  
associated with differentiation of immature precursors of osteoclast precursor cells to  
osteoclasts. ~~These findings have led to the completion of the present invention and~~

maturation thereof. ~~Further investigation into the protein of the~~ The present invention has  
proven been completed based on this finding. Moreover, as a result of further studying  
the protein of the present invention, the present inventors have found that this the novel  
membrane protein is an important protein ~~controlling~~ which leads the differentiation and  
maturation of ~~osteoclasts from immature precursors of osteoclast precursor cells to~~  
osteoclasts by osteoblast-like stromal cells in a co-culture system of the  
~~osteoblastic~~ osteoblast-like stromal cells and spleen cells. ~~The success in~~ successful  
~~identification and~~ isolation and purification of the protein ~~which functions as a factor~~  
~~supporting or promoting~~ which supports and promotes the differentiation and maturation  
of osteoclasts in the present invention ~~has enabled~~ enabled a screening ~~for~~ of a novel  
~~medicine useful for abnormality of~~ agent for treating bone metabolism abnormality, based  
on a mechanism for bone metabolism utilizing in a living subject, using the protein of  
the present invention.

~~Accordingly~~ Therefore, an object of the present invention is to provide a novel  
protein (OCIF- binding molecule ~~or~~ OBM), which binds ~~to~~ osteoclastogenesis inhibitory  
factor (OCIF), and a method ~~to produce this protein for the production thereof.~~ Another  
object of the present invention is to provide DNA ~~encoding this~~ which encodes the  
protein, ~~proteins containing a~~ protein having an amino acid sequence encoded by ~~this~~  
DNA, a method for genetically producing ~~this~~ protein ~~utilizing genetic engineering~~  
~~techniques,~~ and a pharmaceutical compositions comprising ~~this~~ protein. Furthermore,  
~~further another~~ object of the present invention is to provide ~~methods~~ an agent for  
preventing and/or treating bone metabolism abnormality comprising the protein.  
Moreover, another object of the present invention is to provide: a method of screening  
substances for: a substance which controls expression of this protein using this the protein  
and the DNA, substances inhibiting a substance which inhibits or regulating ~~modifies~~ the  
biological activity of ~~this~~ protein, ~~receptors transducing~~ or a receptor which binds the  
action protein and transmits the activity of the protein by binding to; a method of using  
the protein, substances and DNA thereof; a substance obtained by the  
screening method; and pharmaceutical compositions ~~which comprises these comprising~~  
the obtained substances. ~~A still further~~ Furthermore, another object of the present  
invention is to provide ~~antibodies against this protein, methods for preparing an antibody~~

to the antibodiesprotein, a method for production thereof, a method for measuring the protein using the antibody, and a medicament (agent; pharmaceutical compositionscomposition) comprising these antibodiesy.

The protein of the present invention ~~has~~shows the following physicochemical properties and biological activity. That is, (a)-Affinity:— the protein specifically binds to the-osteoclastogenesis inhibitory factor (OCIF) and exhibitshas high affinity to-OCIF-(a dissociation constant, a Kd value, on a cell membrane: Kd=surface, is not larger than  $10^{-9}$  M-or less) ; (b)-Molecular weight:— has the protein shows a molecular weight of approximatelyabout 30,000- to 40,000 whenas determinedmeasured by SDS-polyacrylamide gel-electrophoresis (SDS-PAGE)-under non-reducing conditions, and shows an apparent molecular weight of approximatelyabout 90,000- to 110,000 when cross-linkedcrosslinked towith a monomer-form-type OCIF; and (c)-Biological the protein has an activity:— exhibits activity-supporting or promoting-osteoclast to support and promote the differentiation and maturation of osteoclasts in a co-culture system-of the mouse osteoblasticosteoblast-like stromal cells and mouse spleen cells in the presence of bone resorption factors such as the active-form of vitamin D<sub>3</sub> and parathyroid hormones (PTH).

~~As a representative in vitro culture system for osteoclastogenesis, a co-culture system of ST2, a-mouse-osteoblastic-derived osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D<sub>3</sub> or PTH is well known as a typical in-vitro culture system for osteoclast formation. The cells expressingthat express the protein of the present invention can be determinedobtained by testingexamining the binding ability of OCIF toa mouse osteoblasticosteoblast-like stromal cells or mouse spleen cells cultured in the presence or absence of the active-form of vitamin D<sub>3-3</sub> to OCIF. The protein of the present invention is specifiedidentified as thea protein which is-induced specifically induced on thean osteoblasticosteoblast-like stromal cells cultured in the presence of an-osteotropic-bone resorption factors such as the active-form of vitamin D<sub>3</sub> or PTH. In-additionFurther, thein proteinconsideration of-the invention can be specified as a protein-exhibiting biological activity-supporting or promoting differentiation and maturation of osteoclasts from the following results.—That is, thefacts that osteoclast formation is inhibited dose-dependently by the-addition of 1-to~~

~~40 ng/ml of OCIF to the above-mentioned co-culture system in the presence of the active-~~  
~~form of vitamin D<sub>3</sub>, the time course of~~ a dose-dependent manner within a range of 1 to  
40 ng/ml of OCIF; that there is an intimate correlation between change in expression of  
~~the protein of the present invention~~ protein induced on the ST2 cells in the presence of the  
~~active-form of vitamin D<sub>3</sub> well-correlates and the change in osteoclast formation with the~~  
~~the passage of time course of osteoclast formation in the co-culture. In addition;~~ that the  
amount of ~~protein of the present invention~~ protein expressed on a ST2 cells  
correlates ~~ponds with to the capability~~ intensity of the ~~cells~~ ability to support the  
osteoclast formation; ~~and that~~ osteoclast formation is completely inhibited by binding  
of OCIF(s) to the ~~protein of the present invention~~ protein on the ST2 cells completely  
suppresses, the protein of the present invention is identified as a protein having biological  
activity (effect) to support and promote the differentiation and maturation of osteoclasts  
formation.

The affinity of the protein of the present invention ~~to~~ for OCIF can be  
~~evaluated~~ assessed by labeling OCIF and ~~examining~~ testing the binding activity of the  
labeled OCIF to the surface of an animal cell membrane. OCIF can be labeled by a  
~~conventional~~ commonly used protein-labeling method such as labeling with a radioisotope  
or fluorescence labeling. ~~Labeling of tyrosine residues with <sup>125</sup>I can be given as a~~  
~~specific~~ For instance, an example of labeling of the OCIF with a radioisotope. Labeling  
is <sup>125</sup>I labeling at a tyrosine residue, and labeling methods such as iodogen method,  
chloramine T method, and enzymatic and enzyme method can be utilized. can be  
employed thereto. The binding ~~of the activity of the thus~~ labeled OCIF to the surface  
~~membrane of an~~ animal cell membrane can be examined in accordance with a  
commonly used method, and the amount of nonspecific binding can be tested measured  
by a conventional method. The addition adding 100 to 400 times excess amounts of  
unlabeled OCIF to the medium used for the binding assay to a concentration, 100 to 400  
times the concentration of labeled OCIF, ensures measurement of non-specific  
binding experiment. The amount of specific binding of OCIF ~~can be~~ is calculated by  
subtracting ~~the amount that~~ of non-specific ~~the nonspecific~~ binding from that of total  
amount of binding of the labeled OCIF. The affinity ~~of the protein (for OCIF) of the~~  
~~present invention~~ protein expressed on the cell membrane to OCIF can be evaluated is

assessed by conducting the amount test with various amounts of the labeled OCIF and analyzing the amount of the specific binding by Scatchard plot. The determined affinity of the protein of the present invention to OCIF is approximately about 100- to 500 pM. Thus, the protein of the present invention is specified/identified by a protein having such high affinity (the dissociation constant on cell membrane, the  $K_d$  value, on a cell membrane is not larger than  $10^{-9}$  M or less) to osteoclastogenesis-inhibitory factor (for OCIF). The molecular weight of OBM can be accessed/is measured by use of gel filtration chromatography, SDS-PAGE, or the like. To measure the molecular weight more accurately, SDS-PAGE is preferred in order to accurately determine the molecular weight. The preferably used, and OBM is specified/identified as a protein having a molecular weight of approximately about 40,000 ( $40,000 \pm 4,000$ ) under reducing conditions.

The protein of the present invention can be obtained from a mouse osteoblastic/osteoblast-like stromal cell line, ST2, a mouse preadipocyte fat cell line, strain PA6, or human osteoblastic/osteoblast-like cell lines, or other concentrated osteoblastic/osteoblast-like cells selected/obtained from mammals such as humans, mice, or rats/mouse and rat. As the And, substances that are required to induce expression of/express the protein of the present invention, osteotropic on these cells may be bone resorption factors such as the active-form of vitamin  $D_3$  (calcitriol), parathyroid hormone (PTH), interleukins (IL)-1, IL-6, IL-11, Oncostatin M, and leukemia inhibitory cell growth inhibiting factor (LIF)-can be given. These As for the amounts of these substances can be added in, it is desirable to use the concentration of  $10^{-8}$  M (active-form of vitamin  $D_3$  and/or PTH), in an amount of  $10^{-8}$  M; the IL-11 and the oncostatin M in amounts of 10 ng/ml (IL-11), or and 1 ng/ml (Oncostatin M), respectively; and the IL-6 is preferably used at a concentration in an amount of 20 ng/ml with 500 ng/ml of IL-6 soluble IL-6 receptor. Preferably, confluent It is preferable to use cells obtained by culturing mouse osteoblastic/osteoblast-like stromal cell line, ST2, cultured in  $\alpha$ -MEM medium to which containing  $10^{-8}$  M of the active-form of vitamin  $D_3$ ,  $10^{-7}$  M of dexamethasone, and 10% fetal bovine fetal serum were added can be used for at least one week until the cells become confluent. Thus cultured cells may be removed and collected by scraping

~~with using~~ a cell scraper ~~or the like~~. ~~The~~ Moreover, the collected cells ~~may~~ can be stored at -80°C until use.

The protein of the present invention can be purified efficiently from the ~~a~~ membrane fractions of the ~~thus~~ collected cells. The membrane fractions can be prepared ~~by in accordance with a conventional common method which is used to prepare intracellular organelle. Various types used for fractionation of protease inhibitors may be added to the organelles. As a buffer solution used for the used in preparation of the membrane fractions~~ fraction, various protease inhibitors may be preferably added. ~~Examples~~ Illustrative examples of the protease inhibitors ~~to be added~~ include serine protease inhibitors, thiol protease inhibitors, and metalloprotease inhibitors, ~~such as PMSF, APMSF, EDTA, oO-phenanthroline, leupeptine, pepstatin A, aprotinin, and a soybean trypsin inhibitor are given as specific examples. A Dounce homogenizer~~ To crush the cells, a ~~polytron~~ Dounce homogenizer, a polythoron homogenizer, an ultrasonicator or a ultrasonic processor ~~the like~~ can be used to homogenize the cells. The cell homogenate ~~is crushed cells can be~~ suspended in a buffer solution containing 0.5 M of sucrose and centrifuged at 600 X g for 10 minutes at 600 x g, ~~so as to separate the cell nuclei and undisrupted cells as precipitate~~ a precipitated fraction. The supernatant is centrifuged ~~After further centrifugation at 150,000 X g for 90 minutes at 150,000 x g to obtain, a membrane fractions~~ fraction can be obtained as ~~precipitate~~ a precipitated fraction. ~~The~~ By treating the thus obtained membrane fraction ~~is treated by with various types of detergents to efficiently solubilize and extract~~ surfactants, the protein of the present invention ~~from existing on the cell membrane can be solubilized and extracted, efficiently. Detergents~~ For solubilization, various surfactants which are ~~commonly conventionally used to solubilize~~ in solubilization of cell membrane proteins, such as CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), Triton X-100, Nikkol, and n-oetyl glycoside ~~octylglycoside~~, can be used. Preferably, ~~The protein of the present invention is preferably solubilized by adding 0.5% CHAPS is added to the membrane fraction protein and agitating the mixture is at stirred~~ 4°C for 2 hours at 4°C to solubilize the protein of the present invention. The sample ~~By centrifuging the thus prepared is centrifuged sample at 150,000 x X g for 60 minutes to obtain, the solubilized membrane fraction can be obtained as a supernatant.~~

The protein of the present invention can be purified efficiently from the thus obtained solubilized membrane fraction ~~with a~~, using an OCIF-immobilized column, gel, or resin coupled with OCIF. ~~The~~ As immobilized ~~the~~ OCIF may to be used in the immobilization, that isolated from ~~at the culture broth~~ solution of human ~~embryonic fetal~~ lung fibroblasts, IMR-90, in accordance with a method described in WO 96/26217 or ~~rOCIF prepared using that obtained by genetic engineering techniques~~ (rOCIF) can be used. This rOCIF can be prepared ~~obtained~~ by introducing ~~incorporating~~ the corresponding human cDNA, rat or mouse cDNA, or rat cDNA into an expression vector ~~according to~~ in accordance with a conventional common method, ~~transducing~~ expressing the ~~constructed vector in~~ OCIF with animal or insect cells such as CHO cells, BHK cells, ~~or and~~ Namalwa cells, ~~or in insect cells to produce rOCIF~~, and then purifying rOCIF ~~it~~. ~~Obtained~~ The thus obtained OCIF has ~~shows~~ a molecular weight of approximately ~~about~~ 60 kDa (monomer-form type) ~~or and~~ a molecular weight of about 120 kDa (dimer-form type). ~~The~~ A dimer-form type OCIF is preferable for ~~preferably used in the immobilization~~. ~~Given as examples of the gels and resins to which~~ As a gel or a resin for immobilizing OCIF is immobilized are, ECH Sepharose SEPHAROSE® 4B, EAH Sepharose SEPHAROSE® 4B, Tthiopropyl Sepharose SEPHAROSE® 6B, CNBr-activated Sepharose SEPHAROSE® 4B, activated CH Sepharose SEPHAROSE® 4B, Epoxy activated Sepharose SEPHAROSE® 6B, activated thiol Sepharose SEPHAROSE® 4B (these are manufactured by products of Pharmacia Co., Ltd.), TSK kgel AF-Epoxy Toyopal TOYOPAL 650, TSK kgel AF-Amino Toyopal TOYOPAL 650, TSK kgel AF-Formyl Toyopal TOYOPAL 650, TSK kgel AF-Carboxy Toyopal TOYOPAL 650, TSK kgel AF-Tresyl Toyopal TOYOPAL 650 (these are manufactured by Tosoh Corporation products of Toso Co., Ltd.), Amino-Cellulofine, CELLULOFINE™ Ccarboxy-Cellulofine CELLULOFINE™, FMP activated Cellulofine CELLULOFINE™, Formyl-Cellulofine formyl-CELLULOFINE™ (these are manufactured by Seikagaku Kogyo products of Sei Kagaku Kogyo Co., Ltd.), Affigel AFFIGEL 10, Affigel AFFIGEL 15, 15 and Affiprep AFFIPREP 10 (these products of Bio-Rad Co., Ltd.) are manufactured by BioRad Co.) available. As columns to which Furthermore, as a column for immobilizing OCIF is immobilized, HiTrap HITRAP® NHS-activated column (Pharmacia Co., Ltd.), TSKgel Tresyl-5PW (Tosoh



~~Corporation Toso Co., Ltd.), etc. or the like can be given used.~~ As a specific example of the method for immobilizing OCIF ~~to a HiTrap with the HITRAP® NHS-activated column (1 ml, Pharmacia Co., Ltd.), the following method can be given is presented.~~ Specifically That is, 1 ml of 0.2 M NaHCO<sub>3</sub>/0.5 M NaCl solution (pH 8.3) solution containing 13.0 mg of OCIF is injected applied to the column and allowed to perform undergo a coupling reaction at room temperature for 30 minutes. Then, after 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0) are sequentially applied to the column. ~~Then, the column is again washed with~~ respectively, 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) is applied again, and then the column is allowed left to stand ~~for one hour at room temperature for 1 hour so as to block inactivate~~ excess active groups. ~~The~~ Thereafter, the column is sequentially washed twice with 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0), and then washed replaced with 50 mM Tris/1 M NaCl/0.1% CHAPS solution buffer (pH 7.5); ~~thereby obtaining a.~~ Finally, an OCIF-immobilized column can be prepared. Using the prepared OCIF-immobilized column. ~~The, gel or resin, the protein of the present invention can be efficiently purified by a OCIF-immobilized column prepared in this manner, or an OCIF-immobilized gel or resin efficiently.~~ It is desirable to add To prevent the proteolysis of the protein of the present invention, the above various above-mentioned protease inhibitors may also be added to the buffer solutions used for their purification of the protein to suppress degradation of the protein of the present invention. The protein of the present invention can be purified by loading After applying the above-mentioned solubilized membrane fraction onto the an OCIF-immobilized column or by mixing the solubilized membrane fraction with the an OCIF-immobilized gel or resin; and eluting subsequently stirring the mixture so as to cause the fraction to be adsorbed, the protein of the present invention can be eluted from the OCIF-immobilized column, gel; or resin with using an acid, various protein -denaturing agents, a cacodylate buffer; and/or the like. It is desirable to use an acid for elution and to neutralize immediately after elution to To minimize denaturation of the protein of the present invention, it is preferable to neutralize the eluate immediately using a base. As the acid an acid buffer solution used for elution, 0.1 M glycine-hydrochloric acid buffer solution (pH 3.0), 0.1 M glycine-

hydrochloric acid buffer solution (pH 2.0), and 0.1 M sodium citrate buffer solution (pH 2.0), and the like can be given used, for example.

The purified protein of the present invention can be further purified by conventional use of a method which is conventionally employed in purification methods used for purification of various of proteins from biological materials and by samples, through various purification methods utilizing operations taking advantages of the physicochemical properties of these protein of the present invention. To concentrate solutions containing a solution of the protein of the present invention, conventional techniques a method which is conventionally used in the protein purification process for proteins such as ultra-filtration, e.g., ultrafiltration, freeze -drying, and salting-out, can be used. Ultra filtration Preferably, ultrafiltration based on centrifugation with Centricon-CENTRICON®-10 (BioRadAmicon Co. ), for example, and the like is preferably used. As Furthermore, as a means for the purification of purification, various techniques methods conventionally utilized for the used in protein purification of proteins, such as using ion exchange chromatography, gel filtration chromatography, hydrophobic chromatography, reversed phase chromatography, and preparative electrophoresis, are and the like can be used in combination. More specifically, it is possible to purify the protein of the present invention can be purified by a combined combination use of gel filtration chromatography with Superose-SUPEROSE®-12 column (Pharmacia Co., Ltd.) and the like and reverse phase chromatography. To detect Moreover, the protein of the present invention in during the purification process, the binding can be detected by analyzing activity of the protein of the present invention to bind the immobilized OCIF is examined or the material bound to the immobilized OCIF is by immuno precipitated precipitation of OCIF-binding substances with an anti-OCIF antibody and analyzed followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

The thus obtained protein of the present invention is useful, due to its activity, as an agent medicaments, e.g., as agents for treating diseases caused by abnormality of bone metabolism abnormality such as osteopetrosis, or as a reagent for research experimental and diagnosis of these diseases diagnostic reagents.

The Furthermore, the present invention further relates provide to DNA encoding which encodes a novel protein (OCIF- binding molecule or, OBM) which binds

to osteoclastogenesis inhibitory factor (OCIF), ~~proteins containing the~~ a protein having an amino acid sequence encoded by this DNA, a method for ~~the preparation of this~~ protein genetically producing a protein which specifically binds OCIF by use of the ~~genetic engineering technique~~ protein, and ~~pharmaceutical compositions~~ an agent for ~~treating bone metabolism abnormality~~ comprising this protein. Furthermore In addition, the present invention ~~provides methods~~ relates to a method for screening ~~substances to~~ regulate ~~a substance which controls~~ expression of OBM, a method for screening ~~substances inhibiting or modifying the biological activity of a substance which binds~~ OBM and inhibits or modifies an effect thereof, ~~or a method for screening receptors~~ transducing the action of a receptor which binds OBM by binding to OBM and transmits an effect thereof, and ~~pharmaceutical compositions which comprises~~ comprising a ~~substances obtained as a result of the~~ these methods for screening.

The novel protein OBM ~~which is encoded by the DNA of the present invention~~ has ~~shows~~ the following physicochemical properties and biological activity. That is, (a) ~~binds the protein specifically to~~ binds osteoclastogenesis inhibitory factor (OCIF), (b) ~~has the protein shows~~ a molecular weight of approximately about 40,000 ( $\pm$  4,000) ~~when as determined~~ measured by SDS-PAGE under reducing conditions, and ~~shows an~~ apparent molecular weight of approximately about 90,000- to 110,000 when crosslinked ~~to with a monomer-form type~~ OCIF<sub>2</sub>; and (c) ~~exhibits the protein has an~~ activity supporting ~~or promoting to support and promote~~ differentiation and maturation of osteoclasts.

Human osteoclastogenesis inhibitory factor (OCIF) ~~which is used as a probe to~~ identify for assessing the properties of OBM in identification of the DNA encoding OBM, the OCIF- binding molecule OBM of the present invention, and ~~to evaluate properties of~~ OBM can be isolated from at the culture broth solution of a human embryonic fetal lung fibroblast cell line fibroblasts, IMR-90, according to in accordance with WO No. 96/26217. Recombinant For isolation and identification of the DNA encoding OBM, recombinant human OCIF, recombinant mouse OCIF, recombinant rat OCIF<sub>2</sub> and the like can also be used ~~for the isolation and identification of the DNA coding OBM~~. These recombinant OCIF ~~proteins can be produced~~ obtained by ~~inserting~~ incorporating the ~~corresponding DNA fragments encoding these proteins into an expression vectors~~ according to conventional methods vector in accordance with a commonly used method,

subsequently expressing inOCIF with animal or insect cells such as CHO cells, BHK cells, ~~or~~and Namalwa cells, ~~or in insect cells,~~ and then purifying themit.

~~As a method~~Methods for isolatingcloning a cDNA ~~encoding a~~which encodes the target protein (cDNA cloning) include, the a method comprising ~~determination~~the steps of determining a partial amino acid sequence of the protein and ~~isolation of~~isolating the target cDNA by hybridization ~~utilizing the~~based on a nucleotide sequence corresponding to the amino acid sequence ~~can be employed~~. Another ~~available method,~~ even in comprises the ease wheresteps of constructing a cDNA library with an expression vector, regardless of whether or not the amino acid sequence of the protein is ~~not~~ known, ~~comprises constructing a cDNA library in a expression vector,~~subsequently introducing the ~~cDNA~~it into cells, and then screening for the presence and absence of expression of the target protein ~~teand~~ isolateing the ~~objective~~desired cDNA (~~expression cloning method,~~ D' Andrea *et al.*: Cell 57, 277- to 285, 1989; Fukunaga *et al.*: Cell 61, 341- to 350, 1990) (expression cloning method). In the expression cloning method, suitable host cells ~~such as bacteria~~bacterial, yeast, animal cells, and the like are selected depending on the objective. ~~In many cases, animal cells are selected~~and used as the host cells ~~for~~according to the purpose. For cloning a cDNA ~~encoding a~~which encodes a protein such as the protein of the present invention ~~which is considered to be present in~~on the surface of animal cell membrane surface. ~~Normally~~as in the present invention, host animal cells ~~showing~~are often used as hosts. Furthermore, hosts with high efficiency for introducing DNA ~~transfection and achieving expression of~~expressing the introduced DNA at high levels are selectedconventionally used. One of ~~such animal~~the cells having such characteristics is the a monkey kidney ~~cells~~cell line, COS-7, used in the present invention. ~~Because~~Since SV40 large T antigen is expressed in the COS-7 cells, plasmids having a plasmid which has a replicator of SV40 can beorigin of replication are present in the cell as an multicopy episome ~~of multiple copies in the cell,~~ so that a high level ~~of~~whereby higher expression is than usual can be expected. ~~In addition~~Moreover, because ~~expression of a target protein by COS-7 cells reaches a~~since the maximum expression level is reached within a few days after the introduction of DNA, ~~the cell is~~COS-7 cells are suitable for rapidquick screening. ~~A~~In combination ~~of this host cell with a plasmid~~ enablessuitable ~~for~~for high expression ~~ensures gene expression of,~~ this host cell enables an

extremely high level of gene expression. The promoter is a factor exhibiting the greatest influence on the expression of a gene on a plasmid is which has the most significant effects on the amount of gene expression. As a promoter: ~~Promoters such as SR $\alpha$~~  suitable for high level of expression, SR $\alpha$  promoter and cytomegalovirus-derived promoters are often used as high expression promoters. ~~To~~ Screening screen methods for cloning the cDNA encoding of the membrane protein by the include expression cloning strategy, ~~screening procedures such as~~ binding method, panning method; ~~or and~~ film emulsion method are used.

The present invention relates to DNA encoding, which encodes the protein (OBM) which specifically binds to OCIF, isolated (OBM), obtained by the a combination of the expression cloning strategy method and the screening by the binding method, to the protein expressed protein therewith, and to a screening of physiologically a biologically active substances using with the DNA or the expressed protein. OBM encoded by the DNA of the present invention can be detected by labeling OCIF and testing subsequently examining the binding activity of the labeled OCIF to membrane the surface of an animal cell membrane. OCIF can be labeled by a conventional method for labeling method protein such as labeling with a radioisotope labeling method or fluorescence labeling method which. An example of labeling OCIF with radioisotope is used for <sup>125</sup>I labeling common proteins. Labeling at tyrosine residues by <sup>125</sup>I can be given as a, and specific example of labeling OCIF with a radioisotope. ~~Labeling methods such as the iodogene include~~ Iodogen method, chloramine T method, and enzymatic method can be utilized. The binding activity of thus labeled OCIF to the surface of an animal cell membrane surface can be tested assessed in accordance with a commonly used method. Furthermore, an amount of nonspecific binding can be measured by conventional methods. ~~The addition adding~~ 100 to 400 times excess amount of unlabeled OCIF to the medium ~~used for the test to a concentration, 100 to 400 times the concentration of labeled OCIF, enables quantification of the amount of non-specific binding experiment~~. The amount of specific binding of OCIF ~~can be~~ is calculated by subtracting that of the amount of non-specific nonspecific binding from that of the total amount of binding of the labeled OCIF.

The present inventors ~~Based on an assumption~~ that there is interaction between the factor, which is involved in differentiation of osteoclasts and OCIF. ~~Based on this assumption, to isolate the protein to which recombinant interacts with~~ OCIF binds, the inventors ~~have screened the~~ expression library prepared from the mRNA of a mouse osteoblastic/osteoblast-like stromal cell line, ST2, ~~according to~~ with recombinant OCIF in accordance with the following method in order to separate the protein which binds OCIF. Specifically, DNA synthesized using from the mRNA of ST2 mRNA cells was inserted into an expression vector for an animal cell cell, and the vector with the insert was introduced they were transduced (transfected) into COS-7 monkey kidney COS-7 cells. The objective Using  $^{125}\text{I}$ -labeled OCIF as a probe, the target protein expressed on the COS-7 cells was screened using OCIF labeled with  $^{125}\text{I}$  as a probe. As a result, DNA encoding which encodes the protein which binds that specifically to binds OCIF was isolated. The could be separated, and then the nucleotide sequence of the DNA encoding which encodes this OCIF- binding molecule (OCIF- binding molecule; OBM) was then determined. Moreover Furthermore, it has been found that OBM encoded by this DNA was found to bind strongly and specifically and strongly to binds OCIF, on the cell membrane.

Comparatively An example of DNA hybridization under relatively mild conditions for hybridization of DNA in the present invention are the conditions, for example, wherein is that after DNA is transferred to a nylon membrane and immobilized thereto according to conventional methods and fixed in accordance with a common method, it is hybridized in a buffer solution for with a radio-labeled DNA as a probe in a hybridization with a probe DNA labeled with an isotope buffer at a temperature of 40- to 70°C for about 2 hours to overnight, followed by and then washed in with 0.5  $\times$  SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes. Specifically More specifically, Highbond after DNA is transferred and fixed to a nylon membrane, HYBOND® N (Amersham Co.) is used as the nylon membrane to transfer and immobilize DNA thereon, Ltd. DNA, in accordance with a conventional method, it is then hybridized with a probe DNA- $^{32}\text{P}$ -labeled with  $^{32}\text{P}$  DNA as a probe in a rapid hybridization buffer Rapid Hybridization Buffer (Amersham Co., Ltd.) at 65°C for 2

hours, followed by and then washing with 0.5 ~~X~~ SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes.

As a representative *in vitro* culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblastic/osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D<sub>3</sub> or PTH is well known as a typical *in vitro* culture system for osteoclast formation. The protein OBM of the present invention is specified as the identified as a protein which is induced- specifically induced on the osteoblastic stromal cells/an osteoblast-like stroma cell cultured in the presence of an agent which accelerates bone resorption factors such as an active-form of vitamin D<sub>3</sub> or and PTH. In addition Furthermore, because of the fact that formation of since osteoclasts formation is stimulated by the addition of adding the protein encoded by the DNA of the present invention to a culture system of mouse spleen cells cultured even in the absence of the active-form of vitamin D<sub>3</sub> or PTH, OBM which is encoded by the DNA of the present invention is considered to be involved in the differentiation and maturation of osteoclasts.

Recombinant A recombinant OBM can be produced by inserting the DNA of the present invention into an expression vector so as to construct/prepare a plasmid for expressing OBM, and then introducing and expressing the plasmid into various cells or microorganisms to express recombinant OBM and microbial strains. As a host in which recombinant OBM is expressed, mammalian cells such as COS-7, CHO, Namalwa, or bacteria such and the like can be used as mammalian hosts cells for expression, and Escherichia coli (E. coli) and the like can be used as bacterial host cells for expression. In such a case, the recombinant OBM may can be expressed as a membrane-bound-form protein using the full length of DNA or as a secretionry-form type or asolubilized-type (soluble-form type) protein by removing a part of the portion DNA encoding thea transmembranemembrane-binding domain from the full length. Thus produced recombinant OBM can be purified efficiently purified using a suitablein combination ofwith conventional methods used in protein purification methods used for common proteins, such as affinity chromatography using an OCIF-immobilized columns, ion exchange chromatography, and gel filtration chromatography and the like. The thus obtained protein of the present invention is useful, due to its activity, as an

agent/medicaments, e.g., as agents for treating diseases caused by abnormality of bone metabolism abnormality such as osteopetrosis or as a reagent for research and diagnosis of such diseases as experimental or diagnostic reagents.

The following screening operations can be carried out using the protein OBM encoded by the DNA of the present invention enables: (1) screening of substances a substance which regulate/controls expression of OBM<sub>2</sub>; (2) screening of substances a substance which specifically bind to/binds OBM and inhibit/inhibits or modifies the biological activity of OBM<sub>2</sub>; and (3) screening of proteins a protein (OBM receptor) which are present in osteoclast/exists on a precursor cell/cell of osteoclasts and transduce/emits the biological activity of OBM<sub>2</sub>; and (OBM receptor<sub>4</sub>). It is also possible to develop as well as developments of antagonists and agonists using this OBM receptor. In the combinatorial chemistry using the above-mentioned OBM or OBM receptor, a peptide library used for the screening of the antagonists required to identify an antagonist or agonists can be prepared by/in accordance with the following method. Specifically, one of the specific methods. One of them is a split method (Lam *et al.*; Nature 354, 82- to 84, 1991). According toIn this method, synthetic carriers (beads) each comprising a specific amino acid (unit) are bound thereto/are prepared to amino acids (units), separately for all units. The synthesized carriersThen, these synthetic beads are mixed altogether/together and divided into portions an equal to the number of the units. Then, the next units are and then bound to the subsequent units. This procedure is repeated "By repeating this operation n" times to produce, a library containing carriers to/in which "n" units are bound to the carriers is prepared. According to this synthetic method, each carrier pool has one type ofSuch an operation allows the synthesis of only one sequence per one group of the carriers. Therefore/Hence, it is possible to identify a peptide specifically binding to the protein of the present invention by selecting the pool which gives a signal when a positive carrier group is selected in this/said method for screening method using/by use of the protein of the present invention; and determining/then the amino acid sequence of the thereof is determined, a specifically binding peptide bound on the pool can be identified. Another/As another method, is a phage display method which utilizes phage carrying can be used. In this method, synthetic DNA which encode genes encoding random peptides with random amino acid sequences are expressed using phage.



~~The~~ While this method has ~~the~~ an advantage of increasing the ~~that it can archive a larger~~ number of molecules in ~~the~~ a library as compared with ~~than~~ the above-mentioned synthetic peptide-library-method, but it also has the ~~a~~ disadvantage that the kind of less variety for a ~~given number of peptides per molecules is not as varied~~ because ~~there can be~~ particular peptides having sequences which are missing that phages don't prefer do not ~~exist~~ in the library ~~if the phages are unable to express those sequences~~. In the phage display method, as in the case of the split method, using a screening system using ~~with~~ the protein of the present invention ~~can also be applied to determine the nucleotide sequence encoding the peptide~~. ~~That is, the phage specifically binding to the protein of the present invention is~~ there to are concentrated by panning, ~~the selected~~. Thus obtained phage is ~~are~~ amplified in E. coli, and further, the nucleotide sequence encoding the peptide is determined. ~~In addition~~ Furthermore, when it is desired that a specific peptide ~~exhibiting high specificity and having high affinity to~~ for OBM or OBM receptor ~~can be~~ is screened from a peptide library using the ~~systems mentioned~~ system of the above in (2) ~~and~~ or (3), a specific peptide having a very high affinity can be obtained by screening a positive carrier or phage in the co-presence of OBM or OCIF while ~~increasing the~~ OBM with a change of concentration of OBM or OCIF. ~~Only positive carrier pools or phages are selected in this manner~~. For example, screening of a peptide agonist of low molecular weight peptide agonists exhibiting ~~having~~ an EPO (erythropoietin)-like activity ~~were screened from a varied peptide library using a receptor of~~ with an erythropoietin (EPO) which is a hematopoietic hormone) receptor, the tertiary analysis of a three-dimensional structure thereof, and the production of this ~~substance was analyzed, and based on this tertiary structure,~~ (agonist) of low -molecular -weight substances (antagonist) exhibiting the ~~having an~~ EPO-like activity were synthesized through synthesis of organic chemical compounds based on the three-dimensional structure has already been successful (Nicholas *et al.*: Science, 273, 458- to 463, 1996).

~~The present~~ Furthermore, the inventors have previously discovered using the ~~osteoclastogenesis inhibitory factor, OCIF,~~ found that an OCIF-binding ~~a~~ protein binding ~~OCIF~~ is specifically expressed on ~~osteoblastic~~ an osteoblast-like stromal cell line, ST2, which was ~~cultured in the presence of a osteotropic bone resorption factors~~ such as the

active-form of vitamin D<sub>3</sub> and parathyroid hormone (PTH), using osteoclastogenesis inhibitory factor (OCIF). Moreover, the inventors further have found that this protein exhibits a biological activity to support or stimulate, which is associated with differentiation or maturation of osteoclasts from immature osteoclast precursor cells, and clarified various to osteoclasts and maturation thereof, has a biological activity as a factor which supports and promotes so-called differentiation and maturation of osteoclasts. After purification of the protein, the physicochemical properties and the biological activity of this protein by purification thereof the protein were examined. In order to compare the inventors have compared the physicochemical properties and biological activity of the recombinant protein OBM expressed by expressing the DNA of the present invention and with those above mentioned of a purified natural -type protein which specifically binds to OCIF, the present inventors investigated the physicochemical properties and biological activities of the two proteins in order to clarify differences between them. As a result, the two proteins were confirmed ① to be they have found that (1) each of both proteins is a membrane-bound proteins which and specifically bind to binds OCIF, ② to have; (2) they shared a molecular weights of approximately about 40,000 determined as measured by SDS-PAGE; and ③ (3) to they have an apparent molecular weights of about 90,000- to 110,000 when cross-linked crosslinked to with a monomer-form-type OCIF. Not only are these, which indicates that they have very similar physicochemical properties identical, but both proteins exhibit a biological. An activity to support and stimulate promote differentiation and maturation of osteoclasts was also shared by them as well. Therefore, suggesting the possibility that these both proteins are the same protein identical was suggested. In addition Furthermore, a rabbit anti-OBM rabbit polyclonal antibody produced using the purified protein prepared by expressing with the protein (recombinant OBM), which was genetically expressed with the DNA of the present invention by a genetic engineering technique (recombinant OBM) was confirmed to and then purified, has cross-react with the above-described reactivity to the purified natural-type protein, to inhibit specific-type protein obtained by the above method and specifically inhibited the binding of this purified between said natural -type protein and OCIF in the same manner, just as the antibody it inhibits specific binding of between OBM and OCIF. Based on From these results, it is clear obvious that the

recombinant protein OBM expressed ~~by~~with the DNA of the present ~~invention~~ is identical to the natural -type protein which specifically binds to OCIF.

~~To isolate~~Furthermore, for isolating a gene (cDNA) encoding that encodes a human-derived OCIF- binding protein molecule (hereinafter ~~called~~referred to as "human OBM") which specifically binds to OCIF and ~~exhibits~~has the an activity to support and ~~stimulate~~promote differentiation and ~~maturation of osteoclasts from mouse spleen cells in the same manner to osteoclasts and maturation, just as the natural -type or the~~ recombinant mouse OBM ~~does, a cDNA library prepared from mRNA derived from human lymph nodes was screened using a human OBM cDNA fragment as a probe. The human OBM cDNA fragment was obtained by~~does, the inventors have carried out a polymerase chain reaction (PCR) ~~in accordance with the method mentioned using primers prepared based on the above using both mouse OBM cDNA prepared from and human lymph node-derived cDNA as a template and the primer which was prepared from mouse~~. Thus, the inventors have screened said cDNA library with the obtained human OBM ~~cDNA~~cDNA fragment. As a result, they have succeeded in isolation of the cDNA ~~encoding which encodes~~ the human-derived protein which specifically binds to OCIF ~~was (human isolated OBM) and determination of the nucleotide sequence of the said cDNA encoding this human OCIF-binding protein molecule (i.e. the cDNA encoding human OBM) was determined. Similar to mouse OBM, this~~They have found that human OBM encoded by the cDNA ~~has characteristics to bind to OCIF strongly and specifically binds OCIF on the a cell membrane and exhibits the has a biological~~ activity to support and promote differentiation and ~~maturation of osteoclasts from mouse spleen cells to osteoclasts and maturation thereof, just as mouse OBM does. Specifically~~That is, other ~~objects of the present invention provides are to provide: (1) DNA encoding which encodes human OBM which is a novel human OBM-derived protein which binds to osteoclastogenesis inhibitory factor (OCIF); a protein which possesses the having an amino acid sequence encoded by the DNA;~~ (2) a method for genetically producing ~~the a~~ protein ~~exhibiting characteristics of which~~ specifically ~~binding to~~binds OCIF and ~~the has an~~ activity to support and promote differentiation and ~~maturation of osteoclasts from mouse spleen cells to osteoclasts and maturation thereof by genetic engineering techniques, pharmaceutical compositions comprising this protein~~use of the DNA; (3) an

agent for the treatment of diseases caused by abnormality of treating bone metabolism; abnormality comprising the protein; (4) a method for screening substances regulating a substance which controls expression of human OBM; (5) a method for screening substances a substance which inhibit binds human OBM and inhibits or modulate the activity of human OBM by binding to it, modifies an effect thereof; (6) a method for screening receptors a receptor which bind to binds human OBM and transmit the action of OBM, transmits an effect thereof; and (7) a pharmaceutical compositions comprising the substances obtained by as a result of these screenings methods for screening.

The present invention further provides relates to DNA encoding which encodes human OBM, a novel human OBM-protein, which specifically binds to OCIF and exhibits has the biological activity to support and promote differentiation and maturation of osteoclasts; a protein which possesses the having an amino acid sequence encoded by the DNA; a method for genetically producing the a protein exhibiting characteristics of which specifically binding to binds OCIF and the has an activity to support and promote differentiation and maturation of osteoclasts by genetic engineering techniques, with the DNA; and pharmaceutical compositions comprising this protein an agent for the treatment of diseases causing abnormality of treating bone metabolism abnormality comprising the protein. Furthermore, the present invention provides also relates to a method for screening substances regulating a substance which controls expression of human OBM; a method for screening substances a substance which inhibit binds human OBM and inhibits or modulate the activity of human OBM by binding to it, a modifies an effect thereof; a method for screening receptors binding to human OBM and transmitting the action of OBM, antibodies against a receptor which binds human OCIF binding protein, OBM and transmits a biological activity of OBM; a pharmaceutical compositions comprising a substance obtained as a result of these antibodies methods for screening; an antibody to the prevention human-derived OCIF binding protein; and an agent for preventing and/or treatment of diseases causing abnormality of treating bone metabolism abnormality using the antibody.

The novel, human-derived OCIF- binding protein molecule-(, human OBM) which is, encoded by the DNA of the present invention has shows the following physicochemical properties and biological activity. That is, (a)-binds human OBM

specifically binds to osteoclastogenesis inhibitory factor (OCIF) (WO 96/26217); (b) has human OBM shows a molecular weight of approximately about 40,000 ( $\pm$  5,000) when as determined measured by SDS-PAGE under reducing conditions and shows an apparent molecular weight of approximately about 90,000- to 110,000 when crosslinked with a monomer-~~form-type~~ OCIF; and (c) exhibits human OBM has a biological activity to support and stimulate promote differentiation and maturation of osteoclasts.

~~Mouse OBM~~ The cDNA which encodes encoding mouse OBM, mouse-derived OCIF- binding protein ~~and used, useful~~ as a probe ~~to for isolate separating~~ and identifying the cDNA ~~encoding which encodes~~ human OBM of the present invention, can be isolated ~~according to the above mentioned method~~ from a cDNA library of a mouse ~~osteoblastic osteoblast-like~~ stromal cell line, ST2. ~~Human~~ Furthermore, human osteoclastogenesis inhibitory factor (OCIF) ~~which is necessary, required~~ to ~~evaluate examine~~ the properties and the biological activity of the protein obtained by ~~expression of expressing~~ human OBM cDNA, can be ~~prepared according to the method described in WO 96/26217 by isolating isolated~~ from ~~at the culture broth solution~~ of human fibroblast cell line, ~~IMP strain IMR-90,90~~ in accordance with the method described in WO 96/26217, or by ~~genetic engineering techniques using~~ it can be genetically produced with the DNA encoding OCIF. ~~Recombinant human OCIF~~ To examine the properties and biological activity of human OBM, recombinant humOCIF, recombinant mouse OCIF, recombinant rat OCIF, or and the like can also be used for the assessment of the properties and biological activity of human OBM. These recombinant OCIFs can be obtained ~~according to conventional methods by inserting cDNA incorporating the corresponding cDNAs into an expression vector vectors in accordance with a commonly used method, expressing the cDNA OCIFs in animal or insect cells such as CHO cells, BHK cells, or and Namalwa cells, or in insect cells, and purifying the expressed proteins them.~~

~~The following methods can be used to isolate~~ Methods for isolating the human cDNA ~~encoding which encodes~~ the target protein (cDNA cloning): include: (1) A method comprising the steps of purifying the protein, determining ~~the a~~ partial amino acid sequence ~~of the protein thereof~~, and isolating the target cDNA by a hybridization using ~~the with~~ DNA fragment having comprising a nucleotide sequence corresponding to the

amino acid sequence as a probe, ② (2) a method applied even in the case where the amino acid sequence (expression cloning method) comprising the steps of the protein is not known, which comprises constructing a cDNA library ~~in~~with an expression vector, regardless of whether the amino acid sequence of the target protein is unknown, introducing the cDNA library ~~them~~ into cells, and screening for the presence and absence of the expression of the target protein so as to isolate the ~~objectivetarget~~ cDNA (expression cloning method); and ③ (3) a method of isolating the cDNA encoding which encodes the target human protein ~~from~~by the hybridization or polymerase chain reaction (PCR) method from cDNA library constructed using ~~from~~ human cells or tissues by hybridization ~~tissue~~ or by the use of polymerase chain reaction (PCR) using the cDNA encoding the which encodes a protein of mammalian origin (derived from a mammal other than human) which possesses and having the same characteristics properties and biological activity as ~~the~~of the human-derived target protein of human origin as a probe; assuming that the cDNA probe has, based on an assumption that the cDNA which encodes the non-human protein shares high homology with the human origin cDNA which that which encodes the desired corresponding human protein to be cloned.

Based on the assumption that human OBM cDNA has a high homology with an assumption that human OBM cDNA is highly homologous with the above mouse OBM cDNA, it is possible to determine which human cells or tissues produceing human OBM can be identified by Northern hybridization method using the latter (mouse OBM) cDNA as a probe. Human OBM cDNA can be cloned as follows. A human OBM cDNA fragment is obtained by the following method using the through PCR using mouse OBM primer prepared from the primers prepared based on the mouse OBM cDNA and the cDNA. Human OBM cDNA fragments can be prepared by the PCR method using cDNA prepared from library of a cell or tissue which produces human OBM-producing tissues such as (e.g., a human lymph nodes node) as identified above, as primers and a template, respectively. These human OBM cDNA fragments are used as probes for screening the The cDNA library of human OBM-producing cells or tissues which were produce human OBM as identified according to the method mentioned above is screened with the human OBM cDNA fragment as a probe, and thus, human OBM cDNA can be obtained. The present invention relates to the DNA encoding human OBM which has

~~characteristics of specific binding to~~ obtained DNA that encodes human OBM, a human-  
derived protein which specifically binds OCIF and ~~exhibits~~ has biological activity to  
support and promote differentiation and maturation of osteoclasts. ~~Because~~ Since  
~~the human OBM which is encoded by the DNA of the present invention is a membrane-~~  
~~bound type protein which comprises~~ having a transmembrane domain, ~~this protein it~~ can  
be detected by labeling OCIF and ~~by examining the~~ then binding of the labeled OCIF to  
the surface of an animal cells in which the cDNA of the present invention ~~was~~ is  
expressed. ~~The above described~~ In such a case, OCIF can be labeled by a method which  
is conventionally used for labeling method using protein such as labeling with a  
radioisotope or fluorescein ~~conventionally applied to and fluorescence labeling proteins~~  
~~can be used for labeling OCIF.~~

The molecular weight of the protein expressed by ~~the human OBM cDNA of the~~  
present invention ~~can be accessed~~ is determined by gel filtration chromatography, SDS-  
PAGE, ~~or and~~ the like. ~~In order to accurately~~ To determine the molecular weight more  
accurately, it is desirable to use the SDS-PAGE method is preferably used, by which and  
human OBM ~~was~~ is specified identified as a protein having a molecular weight of  
~~approximately about~~ 40,000 (40,000  $\pm$  5,000) under reducing conditions.

~~Comparatively~~ An example of DNA hybridization under relatively mild conditions  
~~for hybridization of DNA in the present invention are the conditions, for example,~~  
~~wherein is that after~~ DNA is transferred to a nylon membrane and ~~immobilized thereto~~  
fixed in accordance to with a ~~conventional~~ commonly used method ~~and, the DNA is~~  
hybridized with another radiolabeled DNA as a probe ~~DNA labeled with an isotope in a~~  
~~buffer solution for hybridization buffer at a temperature of 40-° to 70°C for about 2 hours~~  
~~to overnight, followed by washing in and then washed with 0.5 \*X SSC (0.075 M sodium~~  
~~chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes. Specifically~~ More  
specifically, Highbond ~~after DNA is transferred and fixed to a nylon membrane, which is~~  
HYBOND® N (Amersham Co., Ltd.) is used as, in accordance with a conventional  
method, the nylon membrane to transfer and immobilize DNA thereon. The DNA is  
~~then~~ DNA is hybridized with another <sup>32</sup>P-labeled DNA as a probe DNA labeled with <sup>32</sup>P  
~~in a rapid hybridization buffer~~ Rapid Hybridization Buffer (Amersham Co., Ltd.) at 65°C

for 2 hours, followed by and then washing with the above 0.5-X SSC at 45°C for 10 minutes.

~~As a representative *in vitro* culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblastic/osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D<sub>3</sub> or PTH is well known as a typical *in vitro* culture system for osteoclast formation. Interaction by adhesion of osteoblastic~~ For promoting osteoclastogenesis in this *in vitro* culture system, both the interaction between a osteoblast-like stromal cells and a spleen cell through their binding, and the presence of an osteotropic bone resorption factors such as the active-form of vitamin D<sub>3</sub> and PTH are indispensable for the osteoclasts formation in this *in vitro* culture system essential. In this *in vitro* culture system, a recombinant COS cells, monkey kidney cells having no osteoclast formation supporting capability, acquire capability to support osteoclasts formation cell strain, resulting from spleen cells in the absence of an osteotropic factor when the expression of the cDNA of the present invention was expressed as osteoblastic thereon, has obtained an ability to support osteoclast formation from spleen cells, just like the osteoblast-like stromal cell line ST2 did, while COS-7 cells (a monkey kidney-derived cell line) does not have an ability to support osteoclast formation in the absence of said bone resorption factors. Based on the fact that Furthermore, since the cDNA of the present invention encodes a membrane-bound protein comprising a transmembrane domain form, this cDNA protein can be expressed as a secretion form type or solubilized form by type protein after removing the part fragment which encodes this transmembrane the membrane binding domain thereof. It was also been confirmed that osteoclastogenesis can be formed was promoted simply by adding the addition of the secretion form secretory-type human OBM to the above-mentioned *in vitro* culture system in the absence of osteotropic said bone resorption factors. Based on From these results, the human OBM which is encoded by the cDNA of the present invention is specified identified as the a factor involved in the differentiation and maturation of osteoclasts.

A recombinant Recombinant human OBM can be prepared by inserting the cDNA of the present invention into an expression vector, preparing so as to prepare a plasmid for expressing human OBM-expression plasmid, and then introducing and



~~expressing the plasmid into~~ various ~~cell~~cells and strains and ~~expressing OBM in the~~ cells. ~~Mammalian~~ cells such as COS-7, CHO, and Namalwa cells, or bacteria such ~~and the like can be used as Escherichia~~ mammalian host cells suitable for expression, and ~~E. coli and the like can be used as a~~ bacterial host for ~~expressing OBM~~cells. In those cases, ~~recombinant human OBM may~~can be expressed as a membrane-bound-form protein, ~~by using the full length of DNA, or as a secretion~~ry-formtype or solubilized-formtype protein by removing a region which encodes the part encoding the ~~transmembrane~~membrane binding domain. ~~The~~Thus produced recombinant ~~human OBM~~ thus produced can be ~~purified~~ efficiently ~~purified using a suitable~~in combination of ~~with~~ conventionally ~~purification used~~ methods used for ~~common~~purifying proteins such as affinity chromatography using OCIF -immobilized ~~columns~~or a column, ion exchange chromatography, and gel filtration chromatography and the like. ~~Human~~Thus obtained ~~human OBM of the present invention thus obtained~~ is useful, due to its activity, as a ~~medicament, e.g., as an agent for treating diseases caused by abnormality of bone metabolism abnormality such as osteopetrosis or as an~~ experimental and diagnostic reagent ~~for research and diagnosis of such diseases.~~

The following ~~screening operations can be carried out using the~~human OBM protein-OBM encoded by the ~~DNA~~AcDNA of the present invention ~~enables~~: (1) screening of ~~substances~~a substance which ~~can regulate~~controls expression of human OBM<sub>51</sub>; (2) screening of ~~substances~~a substance which specifically ~~bind to~~binds human OBM and inhibits or modifies the biological activity of ~~human OBM~~<sub>51</sub>; and (3) screening of a human ~~proteins~~protein (human OBM receptor) which ~~are present~~exists in osteoclasta precursor ~~cell~~cell of human osteoclasts and transmits the biological activity of human OBM (~~human OBM receptor~~). It is also possible to ~~develop~~ antagonists, as well as ~~development of~~ antagonist and agonists using this human OBM receptor. In the combinatorial chemistry using the ~~above~~ human OBM or human OBM receptor, a peptide ~~libraries~~requiredlibrary, which is employed for the ~~screening~~identification of ~~antagonists~~an antagonist or ~~agonists~~agonist, can be ~~produced by~~prepared in accordance with the same method as ~~used for the screening using the~~ mouse OBM. ~~A~~After screening the peptide ~~with~~extremely library by said method in which human OBM is used instead

of mouse OBM, a specific peptide having very high specificity and affinity can be obtained by screening peptide libraries using human OBM instead of mouse OBM.

Although this OBM is very Furthermore, for measurement of OBM, a highly useful as protein mentioned described above and antibodies, it is necessary to obtain an antibody which specifically recognizes OBM and establish an enzyme immunoassay using these antibodies are indispensable in determination of OBM concentration it. However, no antibodies sy useful for the access measurement of OBM concentration have has been so far available. In addition Moreover, an anti-OBM antibody or anti-sOBM antibody which neutralizes the biological activity of OBM or sOBM is supposed assumed to suppress the an activity of OBM or sOBM, specifically the activity to induee promote osteoclasts formation. These, are and expected to be useful developed as therapeutic agents to treat abnormality of an agent for treating bone metabolism abnormality. However, no such antibodies have so far an antibody has not been available.

In view of this situation Under that circumstance, the present inventors have conducted extensive made intensive studies. As a result, the present inventors and have found antibodies (anti-OBM/sOBM antibodies) which recognize both OBM of the following antigens, a membrane-bound protein (OCIF binding molecule; OBM) which specifically binds to osteoclastogenesis inhibitory factor (OCIF); and a soluble-type OBM (sOBM) which lack a transmembrane lacking the membrane binding domain. Accordingly Therefore, objects of the present invention provides antibodies are to provide: (1) an antibody (anti-OBM/sOBM antibodies) which recognizes both OBM of the following antigens, a membrane-bound protein (OBM) which specifically binds to osteoclastogenesis inhibitory factor (OCIF); and soluble OBM (sOBM which lack a transmembrane) lacking the membrane binding domain; (2) a method for the preparation production thereof; (3) a method for determination of measuring OBM and the sOBM concentrations using these antibodies by use of said antibody; and agents (4) an agent for the prevention preventing and/or treatment of diseases resulting from abnormality of treating bone metabolism abnormality which comprises said antibody as an active ingredient.

The present invention relates to antibodies: (1) an antibody (anti-OBM/sOBM antibodies) which recognizes both of the OBM following antigens, a membrane-bound

protein (OCIF binding molecule; OBM) which specifically binds to osteoclastogenesis inhibitory factor (OCIF); and a soluble-type OBM (sOBM) which lack a transmembrane lacking the membrane binding domain; (2) a method for the preparation/production thereof; (3) a method for quantifying/measuring OBM and the sOBM using these antibodies by use of said antibody; and agents (4) a pharmaceutical composition comprising said antibody as an active ingredient, particularly, an agent for the prevention/preventing and/or treatment of diseases resulting from abnormality of treating bone metabolism abnormality. The antibodies

An antibody of the present invention exhibits an antibody which has an activity of neutralizing the to neutralize osteoclastogenesis accelerating/promoting activity, which is the biological activity that OBM and sOBM have, said antibody has any of OBM and sOBM and comprises the antibodies having the following characteristics/properties: (a) a polyclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (anti-mouse OBM/sOBM polyclonal antibody); (b) a polyclonal antibody which recognizes both human OBM and human sOBM antigens (anti-human OBM/sOBM polyclonal antibody); (c) a monoclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (anti-mouse OBM/sOBM monoclonal polyclonal antibodies); (d) a monoclonal antibody which recognizes both human OBM and human sOBM antigens (anti-human OBM/sOBM monoclonal polyclonal antibodies); and (e) an anti-human OBM/sOBM monoclonal antibody which crossreacts has crossreactivity to both mouse OBM and mouse sOBM antigens.

The polyclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (hereinafter called/referred to as “anti-mouse OBM/sOBM polyclonal antibody”) and the polyclonal antibody which recognizes both human OBM and human sOBM antigens (hereinafter called/referred to as “anti-human OBM/sOBM polyclonal antibody”) were produced/can be obtained by the following method/means. The A purified mouse OBM used as an antigen for immunization can be obtained according to in accordance with the above-mentioned method. Especially/That is, natural-type mouse osteoblastic OBM can be obtained by treating a mouse osteoblast-like stromal cell line, ST2, was treated with the active-form of vitamin D<sub>3</sub> and OBM on subsequently purifying it from the cell membrane was purified using membranes of said cell by

~~means of OCIF- immobilized on a column and gel filtration chromatography, thereby obtaining natural mouse OBM (native OBM). The~~ Alternatively, after incorporating the above-mentioned mouse OBM cDNA (Sequence Table, Sequence No. SEQ ID NO: 15) or human OBM cDNA (Sequence Table, Sequence SEQ ID No. NO: 12) was inserted into an expression vector according to conventional methods. Recombinant mouse OBM (Sequence Table, Sequence ID No. 1) and recombinant human OBM (Sequence Table, Sequence ID No. 11) can be obtained by expressing cDNA OBM in an animal cell or insect cell such as a CHO cells, a BHK cells, Namalwa, or a COS-7 cells, insect cell or *Escherichia-E. coli*, and then purifying them using by the same purification methods method as mentioned described above. These, recombinant mouse OBM (SEQ ID NO: 1) or recombinant human OBM (SEQ ID NO: 11) can be obtained, and these may also be used as antigens for immunization. In At this instance time, purifying it takes tremendous effort to highly purify a large amount and a high level quantity of mouse OBM or human OBM which are, a membrane-bound proteins is a task requiring a great deal of labor protein (OBM). On the other hand, as mentioned above, OBM which it has been confirmed that there is no difference in ability to promote differentiation and maturation of osteoclasts between OBM, a membrane-bound protein, and a soluble-type OBM (sOBM), which is a soluable protein obtained by deleting transmembrane the membrane binding domain of OBM are known to be almost the same in their osteoclast differentiation and maturation activities as described above. It is possible to use Accordingly, taking into account that expression and high purification of mouse sOBM and human sOBM which are relatively easily expressed and purified to a high level easy, these sOBMs, solubilized proteins, may be used as antigens for immunization. Mouse sOBM (Sequence Table, Sequence SEQ ID No. NO: 16) and human sOBM (Sequence Table, Sequence SEQ ID No. NO: 17) can be obtained by adding a nucleotide sequence encoding, which encodes a known signal sequence originating derived from the other secretion protein in the secretory-type proteins, 5' upstream side of the 5' end of, respectively, mouse sOBM cDNA (Sequence Table, Sequence SEQ ID No. NO: 18) and or the human sOBM cDNA (Sequence Table, Sequence SEQ ID No. NO: 19), inserting these incorporating the cDNA into an expression vector by the use of genetic in accordance with the same gene engineering techniques, causing these proteins to be

~~expressed in host cells such as various~~method as described above, expressing the protein in a variety of animal cells, insect cells, or ~~E. coli~~ *E. coli* as a host, and then purifying the resultant products. The antigens~~Thus obtained antigen~~ for immunization thus obtained~~are~~ is dissolved in a phosphate buffered saline solution (PBS) and, if necessary, mixed with the~~an~~ same~~equal~~ volume of Freund's complete adjuvant to emulsify~~and~~ emulsified. Then, an animal is immunized with the solution~~if required, and~~ subcutaneously administered to animals about once~~emulsion~~ through a week to immunize these animals~~several~~ few times of subcutaneous administration with a one-week interval between each. A booster injection is given when the~~The~~ antibody titer is measured. When the value reaches a maximum.~~Exsanguination,~~ booster administration is performed. On the 10-days-after<sup>th</sup> day from the booster administration, all the blood was collected. The resulting~~obtained~~ antiserum is treated~~fractionated and precipitated~~ with ammonium sulfate~~precipitation~~. IgG, and the globulin fraction is purified using~~with an~~ anion exchange chromatography or the antiserum is diluted twice with Binding Buffer (Bio-Rad Co., Ltd.) and the diluted antiserum is purified by pProtein A- or pProtein G-Sepharose SEPHAROSE® (Pharmacia Co., Ltd.) column chromatography~~after diluting the antiserum two fold with Binding Buffer™ (BioRad Co.)~~ Thereby, to obtain the desired anti-mouse or anti-human OBM/sOBM polyclonal antibody can be obtained.

The monoclonal antibodies of the present invention can be obtained according to the following method. In the same manner~~That is, as in the case of the polyclonal antibodies,~~an antigen for immunization required to prepare the monoclonal antibody, a natural-type mouse OBM (~~native OBM~~), recombinant mouse or human OBM, or recombinant mouse or human sOBM can be used, as immunogens to prepare monoclonal antibodies used in preparation of the above polyclonal antibody. Hybridomas are produced according to conventional methods by immunizing~~Lymphocytes derived from immunized~~ mammals with these~~each~~ antigens or that obtained by immunizing lymphocytes *in vitro* and fusing the immunized cells~~methods are fused~~ with a myeloma cell~~cell~~ line, and hybridomas are prepared in accordance with a conventional method. By analyzing~~From the hybridoma culture supernatant thus obtained of this hybridoma,~~ a hybridoma producing an antibody which recognizes each antigen is selected by a solid - phase ELISA~~method, antibody producing hybridomas recognizing the,~~ using each highly

purified antigen are selected. The resulting obtained hybridomas are is cloned, and established as a thus obtained stable antibody-producing hybridoma clones. These hybridomas are is cultured to obtain. The target antibody can be obtained therefrom. For preparation of the antibodies. Small mammalshybridoma, immunizing a mammal, a small animal such as micea mouse or ratsrat, are is commonly used to produce hybridomas. Animals are immunized by intravenously or intraperitoneally injecting the antigen diluted to a suitable concentration using a suitableTo immunize the animal, a method comprising the following steps is conventinally used: diluting the antigen with an appropriate solvent such as physiological saltine solution. Optionally to an appropriate concentration and then administering the solution and, if necessary, co-administering Freund's complete adjuvant maybe used together with antigen, into vein (i. These are usually injectedv.) or the abdominal cavity (i.p.), about 3- or 4 times, once in total with a 1 to 2-week or every two weeksinterval between each. The immunized animals are is dissected three dayson the 3<sup>rd</sup> day after finalthe last immunization. Splenocytes, and spleen cells are obtained from the removed isolated spleen are and used as immunocytes (immunized cells). AsIllustrative examples of mouse-derived myeloma to be for cell fusedion with immunized cells,the immunocytes include p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, FO,F0, P3x63 Ag8.653,8. 653 and S194 can be given.194. AFurthermore, illustrative examples of rat-derived cells include cell lines such as R-210 is given as the cell of rat origin.210. Human antibodies are produced by immunizingFor producing human antibody, human B lymphocyteslymphocyte cells are immunized in vitro and fusing the immunized cellsfused with human myeloma cells or a cell line transformed withby EB virus. The fusionFusion of thean immunized cells andcell with a myeloma cells can be carried outcell line is performed according to a conventionalknown methods such as the methodthat of Koehler and Milstein *et al.* (Koehler *et al.* *al.*: Nature 256, 495-497 ( to 497, 1975)). A, while an electric pulse method using an electric pulse ismay also applicablebe used. Immunized lymphocyteslymphocyte cells and myeloma cellscell lines are mixed together at a ratio conventionally acceptedratio used and fused in an FCS free (fetalcommon bovine fetal serum (FCS)-free) medium for cell culture medium with an addition ofin which polyethylene glycol is added. Then, and culturedculture is carried out in an FCS-containing HAT selectionve medium so as to

select a fused cells (hybridomas). Next~~Then~~, ~~the a hybridomas which produce~~ producing an antibodiesy were is selected by using ~~a conventional antibody detection~~ commonly used method for detecting antibody such as ~~an ELISA, a plaque technique, Ouchterlony method, ouchterlony method or aggregation~~ condensation method. Thereafter, to ~~establish stable hybridomasa~~ hybridoma is established. The hybridomas ~~established in this way~~ hybridoma can be subcultured by ~~a conventional~~ common method for culture method or and can be stored by ~~freezing as required in a frozen state if necessary~~. A~~The~~ hybridoma ~~can~~ may be cultured ~~by in accordance with a conventional~~ conventionally used method to collect the culture supernatant or implanted transplanted in the abdominal cavity of ~~mammals to obtain the mammal~~. The antibody can be collected from the ~~ascitic fluid~~ resulting culture solution or ascites, respectively. The antibody in the culture ~~supernatantsolution or ascitic fluid~~ ascites can be purified by ~~a conventional~~ commonly used method such as a salting -out method, ion exchange ~~and~~ chromatography, gel filtration chromatography, or ~~p~~Protein A or ~~p~~Protein G affinity chromatography. Almost all the monoclonal antibodies obtained by the above-described method using sOBM as an antigen are antibodies which can specifically recognize not only sOBM but also OBM (~~such antibodies are hereinafter called~~ referred to as “anti-OBM/sOBM monoclonal antibodiesantibody”). These antibodies can be used for ~~the quantification of OBM or sOBM~~. ~~The amounts~~ measurements of OBM and sOBM ~~can be quantified by labeling~~. After these antibodies are labeled with a radioisotope or an enzyme and ~~by applying the labeled antibodies~~ thus employed to a quantification system measurement systems known such as ~~aas~~ radioimmunoassay (RIA) or enzymeimmunoassay and enzyme immunoassay (EIA), an amount of OBM and sOBM can be measured thereby. ~~Using~~ By use of these quantification measurement systems, ~~thean~~ amount of sOBM in a biological living sample such as blood or urine can be ~~determined~~ measured with ease ~~at and with~~ high sensitivity. ~~In addition, the~~ Furthermore, by use of these antibodies, an amount of OBM ~~binding~~ bound to the surface of a tissue or ~~surface of cells~~ cell can be measured through a binding assay or the like with ease ~~at and with~~ high sensitivity utilizing a binding assay using these antibodies.

When ~~an~~ the obtained antibody is used as a medicament~~ie~~ for humans, it is ~~desirable to use~~, in consideration of a problem of antigenicity, that a human-type anti-

human OBM/sOBM antibody ~~in view of antigenicity is prepared~~. The human-type anti-human OBM/sOBM antibody can be prepared according to by the following methods ①, ②, or ③. ~~In the method ①~~ That is, (1) human lymphocytes collected lymphocyte cells extracted from human peripheral blood or the spleen are ~~immunized~~ sensitized *in vitro* with ~~an antigen-human OBM or or human sOBM in vitro~~ as an antigen in the presence of IL-4. ~~The resulting immunized~~ 4, and then the sensitized human lymphocytes lymphocyte cells are fused with K<sub>6</sub>H<sub>6</sub>/B<sub>5</sub> (ATCC CRL1823), which is a hetero-hybridoma of mouse and human, ~~and thereby, a hybridoma producing the desired antibody is screened to obtain the objective. An antibody-producing hybridoma. The antibodies produced by the resulting antibody-producing hybridomas are from the obtained hybridoma is a human -~~ type anti-human OBM/sOBM monoclonal antibodiesy. ~~The antibodies neutralizing~~ Among these antibodies, an antibody which neutralizes the activity of human OBM/sOBM ~~are is~~ selected from these antibodies. However, ~~in general, it is usually~~ difficult to ~~produce obtain~~ an antibody exhibiting having high affinity to for an antigen by the through such a method of immunizing sensitizing human lymphocytes lymphocyte cells in vitro. Therefore, ~~in order to obtain for obtaining a monoclonal antibodiesy with having high affinity to for human OBM and sOBM, it is necessary to increase the affinity of the human type modify an anti-human OBM/sOBM monoclonal antibodies obtained by the antibody with low affinity as described above method. This can to be done according to the following method that with high affinity~~. First, ~~a~~ A random mutation is introduced into a CDR region (~~particularly CDR-3 region in particular~~) of a said human-type anti-human OBM/sOBM monoclonal antibody which neutralize OBM but have a with low affinity, and make the which a neutralizing antibody obtained as described above. This is expressed with phage to express protein. Phages which ~~can~~ strongly bind to human OBM/sOBM ~~which has the antigen~~ are selected by a phage display method using plates on a plate in which human OBM/sOBM antigens are is immobilized. The ~~selected phages are grown~~ phage is allowed to proliferate in Escherichia *E. coli*. ~~The, and the deduced amino acid sequence of the CDR which exhibits having high affinity is determined from based on the nucleotide sequence of the DNA cloned in the phage thereof~~. The ~~thus obtained DNA encoding gene which encodes the human -type anti-human OBM/sOBM monoclonal antibodiesy is introduced into incorporated and expressed in a~~



commonly conventionally used expression vector for mammalian cells to produce, and then human-type anti-human OBM/sOBM monoclonal antibodies can be obtained.  
Among them, the desired human -type anti-human OBM/sOBM monoclonal antibodies.  
The target human-type anti-human OBM/sOBM monoclonal antibodies exhibiting high affinity and capable of neutralizing antibody which neutralizes the biological activity of human OBM/sOBM and has high affinity thereto can be selected from these monoclonal antibodies.  
In the method ② Furthermore, mouse type anti-human OBM/sOBM monoclonal antibodies are produced according to the same method as in the present invention(2) using BALB/c mouse, an anti-human OBM/sOBM mouse monoclonal antibody is prepared according to a conventionally used method (Koehler *et al.*: Nature 256, 495-49, to 497, 1975) as in the present invention, and a monoclonal antibodiesy which can neutralize neutralizes the biological activity of human OBM/sOBM and exhibiting has high affinity are thereto is selected. These high affinity mouse anti-human OBM/sOBM monoclonal antibodies can be converted into human type using the By CDR- grafting technique method (Winter and Milstein: Nature 349, 293- to 299, 1991) by implanting its, that is a method in which a CDR regions (CDR-1, 2 and 3) of the anti-human OBM/sOBM mouse monoclonal antibody with high affinity are transplanted into the CDR regions of human IgG, a humanized antibody can be obtained. In the method ③ Moreover, (3) human peripheral blood lymphocytes lymphocyte cells are implanted transplanted into a severe combined immune deficiency (SCID) mouse. Because the implanted Thus transplanted SCID mouse can produce produce a human antibodiesy (Mosier D. E. *et al.*: Nature 335, 256- to 259, 1988; Duchosal M. A. *et al.*: Nature 355, 258- to 262, 1992), lymphocytes which can produce the human monoclonal antibodies having specificity to human OBM/ sOBM can be collected by screening SCID mouse immunized. The cells are sensitized with human OBM or sOBM as an antigen and screened. The resulting lymphocytes Thereafter, a lymphocyte cell which produces a human-type monoclonal antibody specific to human OBM/sOBM can be extracted from the mouse. Then, as in the case of the above method for preparing a human-type antibody (1), the obtained lymphocyte cells are fused with K<sub>6</sub>H<sub>6</sub>/B<sub>5</sub> (ATCC CRL1823) which is a heterohybridoma, a hetero hybridoma of mouse and human, according to and then the obtained hybridomas are screened. Then, a hybridoma which

produces the target human-type monoclonal antibody can be obtained. By culturing the thus obtained hybridoma, the procedure described above for the target human-antibodies in the method ①. The resulting hybridomas are screened to obtain hybridomas which can produce the objective human-type monoclonal antibodies. The thus obtained hybridomas are cultured to produce antibody can be produced in large quantities. After purifying them in the same manner as described above, large amounts of the objective human monoclonal antibodies. The antibodies pure products thereof can be purified by the above-mentioned purification method obtained. In addition, it is possible to produce Furthermore, a recombinant human-type monoclonal antibodies antibody can be produced in large amounts quantities by constructing a cDNA library from the said hybridoma which can produce produces the objective human monoclonal antibodies to obtain a gene (cDNA) encoding the objective target human-type monoclonal antibodies by antibody, cloning the cDNA which encodes the target human-type monoclonal antibody, in incorporating this gene said cDNA into an suitable appropriate expression vector by using genetic engineering techniques gene engeneering, and expressing the monoclonal antibodies antibody in host cells such as various a variety of animal cells, insect cells; or *Escherichia coli*; as a host. A large amounts of purified human monoclonal antibodies can be obtained by purifying from the resulting After purification of the antibody from said culture supernatant by the purification methods mentioned above according to the method as described above, a large amount of pure human-type monoclonal antibody can be obtained.

The antibodies which can neutralize Among the anti-OBM/sOBM monoclonal antibodies obtained by the above method, moreover, an antibody which neutralizes the biological activity of OBM/sOBM can be obtained from the anti-OBM/sOBM monoclonal antibodies produced according to this method. ~~The~~ These antibodies which neutralize the biological activity of OBM/sOBM are expected to be useful as medicaments, particularly agents for the treatment preventing and/or prevention of treating bone metabolism abnormality because of their capability of blocking in vivo, since they can inhibit the biological action (an activity of OBM/sOBM, specifically the capability of preventing the induction to promote osteoclast formation) of OBM/sOBM in a living body. The activity of the anti-OBM/sOBM antibodies to neutralize the

biological activity of OBM or sOBM can be measured by determining the determined as an activity to suppress/inhibit osteoclast formation in the *in vitro* system for examining osteoclast formation. Specifically, as *in vitro* assay systems, the following three methods can be used. That is, *in vitro* culture systems for examining osteoclastogenesis culture system can be given include: ① (1) a co-culture system of a mouse osteoblastic/osteoblast-like stromal cell strainline, ST2-cells, 2, and mouse spleen cells in the presence of the active-form of vitamin D<sub>3</sub> and dexamethasone; ② (2) a co-culture system comprising in which OBM expressing is expressed on a monkey kidney cell strainline, COS-7, immobilizing the OBM-expressing cells and fixed with formaldehyde, and culturing mouse spleen cells on those then mouse spleen cells are cultured on the cells in the presence of M-CSF; and ③ a culture system of mouse spleen cells; and (3) a system of culturing mouse spleen cell in the presence of recombinant sOBM and M-CSF. The; however, other systems can be also used. When an anti-OBM/sOBM antibody is added to such a culture system in various concentration and its effect on osteoclastogenesis-inhibitory is examined, an activity of the anti-OBM/sOBM antibodies can be measured by adding the anti of the anti-OBM/sOBM antibodies at various concentrations to these culture systems and investigating their effects on osteoclast formation. The antibody to inhibit osteoclastogenesis-inhibitory can be measured. Also, the activity of of the anti-OBM/sOBM antibodies can also be evaluated by measuring their-OBM/sOBM antibody to inhibit osteoclastogenesis can be determined as an activity to suppress bone resorption-inhibitory activity utilizing *in vivo* using an experimental animals in-vivo. Especially, ovariectomized animal model is given as an-. That is, there is an animal model with progressive osteoclast formation. The osteoclastogenesis-inhibitory activity of the anti-OBM/sOBM antibodies can be determined by administering the, an ovariectomized mouse, in which osteoclastogenesis is increased. An anti-OBM/sOBM antibodies to such -OBM/sOBM antibody is administered to such a kind of experimental animals and evaluating the suppression of animal, and an activity to inhibit bone resorption-(a (an activity to reinforce bone mineral density-increasing activity) is measured. Thereby, an activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be determined.

The thus-obtained antibodies capable of neutralizing antibody, which neutralizes the OBM/sOBM biological activity are of OBM/sOBM is useful in as a medicament,

particularly as a pharmaceutical composition, particularly pharmaceutical compositions to prevent composition for preventing and/or treating bone metabolism abnormality, or as antibodies for an antibody used in immunological diagnosis of such diseases a disease. The preparations antibody of the present invention can be prepared in a formulation, and administered orally or parenterally. A formulation comprising the antibodies of the present invention can be administered either orally or non-orally. Such preparations can be safely administered to humans or animals as a pharmaceutical composition comprising the antibody which contain the antibodies recognizing recognizes OBM and/or sOBM as an active component ingredient. As the forms Illustrative examples of the formulation of the pharmaceutical composition, injection agents including intravenous include injectable solutions such as drip, suppository agents, nasogastric agent, sublingual agents, percutaneous absorption agents, agent and transdermal agent. Since the like are given. Because monoclonal antibodies are macromolecule proteins, they not only readily adhere antibody has a high molecular weight, its adsorption to a glass containers such as a vial or a and syringe tube is significant. Furthermore, but also are the antibody is unstable and easily denatured by inactivated due to various physicochemical factors such as heat, pH, or and humidity. Therefore Thus, to stably formulate the preparations should be stabilized by the addition of stabilizers antibody, stabilizer, pH adjusters, buffering agents buffer, solubilizing agents, or detergent surfactant and the like are added thereto. As Illustrative examples of the stabilizers, stabilizer include amino acids such as glycine and alanine, saccharides such as dextran 40 and mannose, and sugar alcohols such as sorbitol, mannitol, and xylitol can be given xylitol. These stabilizers may be used either individually or in combinations of two or more. The amount of These stabilizers to be added is are preferably from added in an amount which is 0.01 to 100 times, particularly preferably from 0.1 to 10 times, as much as the amount weight of the antibody. The By addition of these stabilizers, increases the storage stability of liquid preparations formulation or lyophilized products thereof freeze-dried formulation can be improved. Phosphate buffers and citrate buffers are given as Illustrative examples of the buffering agents buffer include phosphate buffer and citrate buffer. The buffering agents not only adjust buffer adjusts the pH of the liquid preparations or an aqueous solutions obtained by reformulation or a

reconstituted solution of freeze-dissolving the lyophilized products thereof dried formulation, but also increase and thereby contributes to the stability and solubility of the antibody therein. It is desirable to add the buffering agent in an The amount to make from of the buffer is preferably, for example, 1 mM to 10 mM concentration of the liquid preparation or of the in an aqueous formulation or a reconstituted solution prepared from the lyophilized product of freeze-dried formulation. Polysorbate The surfactant is preferably polysorbate 20, Pulluronic PLURONIC® (BASF Co., Co.) F-68, 68 and polyethylene glycol are given as examples of the detergent. A, particularly preferred example is Polysorbate 80, preferably polysorbate 20. These detergents may be used either individually or in combinations of two or more. Macromolecule proteins such as A protein having high molecular weight like an antibody is easily adherable to adsorb to glass containers or resin, which a container is made of. Adherence to containers However, by addition of a surfactant adsorption of the antibody in a liquid preparation or to a container in an aqueous formulation or a reconstituted solution prepared by reof freeze-dissolving a lyophilized product dried formulation can be prevented by adding such detergents at a concentration from. The surfactant is preferably added in an amount of 0.001 to 1.0% of the weight of an aqueous formulation or a reconstituted solution of freeze-dried formulation. The preparations formulation comprising the antibodies of the present invention can be obtained prepared by adding stabilizers addition of the stabilizer, buffering agents, or agents which prevent buffer and adsorption-preventing agent as described above. When the preparations are Particularly, when it is used as injection agents an injectable formulation for medication medical applications or for treating animals, such injection agents should preferably have an acceptable osmotic pressure ratio of is preferably 1 to or 2. The osmotic pressure ratio can be adjusted by increasing or decreasing the amount of sodium chloride when making the preparations in formulation. The amount content of an the antibody in a the preparation formulation can be suitably adjusted depending appropriately, dependent on the disease to be treated with said formulation, route of administration, route and the like. A The dose of a the human-type antibody administered to humans may be changed depending depends on the affinity of the antibody to human OBM/sOBM, especially, that on is, the dissociation constant (Kd value) of the antibody to human OBM/sOBM. The higher the affinity is (or the lower

the K<sub>d</sub> value is, the smaller the K<sub>d</sub> value), the less the dose that is required to be administered to humans to obtain a certain exhibit medicinal effect/benefits. Because a human-type antibody has a long half-life time of human-type antibodies in human blood of is about 20 days, it is sufficient to administer it the human-type antibody can be administered to humans at in a dose of about 0.1- to 100 mg/kg at least once or more in a within 1- to 30 day period/days, for example.

## BRIEF DESCRIPTION OF THE DRAWINGS

FigureFig. 1 shows the results of SDS-PAGE of mouse OBM protein of Example 3 of the present invention obtained in Example 3, wherein:

<Explanation of symbols>

- (A): Lane 1: Mmolecular weight markersmarker,  
Lane 2: —A partially purified sample (fraction eluted with Gly-HCl (pH 2.0)-elution fraction) obtained, which was derived from ST2 cells cultured in the presence of the active-form of of vitamin D<sub>3</sub> and dexamethasone.,  
Lane 3: —A partially purified sample (fraction eluted with Gly-HCl (pH 2.0)-elution fraction) obtained, which was derived from ST2 cells cultured in the absence of the active-form of of vitamin D<sub>3</sub> and dexamethasone.,  
(B): Lane 1: Mmolecular weight markersmarker,  
Lane 2: Mmouse OBM protein (Example 3) of the present invention after purification by reverse purified with reversed phase high performance liquid chromatography (Example 3).

FigureFig. 2 shows the results of the binding assay/experiment of the <sup>125</sup>I -labeled OCIF to osteoblastic/an osteoblast-like stromal cells, ST2, in Example 4.

FigureFig. 3 shows the osteoclast genesis-formation capability-supporting abctivity of osteoblastic/osteoblast-like stromal cellcell line, ST22, fromwith different generations passage numbers, in Example 5(1), wherein:

<Explanation of symbols>

- 1: Ability osteoclastogenesis-supporting activity of ST2 cells from aboutwith a passage number of around 10<sup>th</sup> subculture to support osteoclast formation.'s,

2: Ability osteoclastogenesis-supporting activity of ST2 cells from about with a passage number of around 40<sup>th</sup>-subculture to support osteoclast formation's.

~~Figure~~Fig. 4 shows a change with the passage of time in expression of the protein of the present invention on the an osteoblast-like stromal cell membrane of osteoblastic stromal, said cells were cultured in the presence of an active-form of vitamin D<sub>3</sub> and dexamethasone, with passage of time, in Example 5(2).

~~Figure~~Fig. 5 shows a change with the passage of time in osteoclast formation osteoclastogenesis in the co-culture system, with passage of time, of Example 5 (2).

~~Figure~~Fig. 6 shows the inhibitory effect on osteoclast formation osteoclastogenesis-inhibiting effects when OCIF was treated with OCIF for different culture only during various culturing periods during in the co-culture period in of Example 5(3).

~~Figure~~Fig. 7 shows the results of a crosslinking test experiment of the <sup>125</sup>I-labeled OCIF with the protein of the present invention, in Example 6-6, wherein:

<Explanation of symbols>

Lane 1: <sup>125</sup>I-labeled OCIF-CDD1,

Lane 2:- sample resulting from crosslinking of <sup>125</sup>I-labeled OCIF-CDD1 crosslinked with ST2 cells1 with an ST2 cell line,

Lane 3:- <sup>125</sup>I-labeled OCIF-CDD1 crosslinked sample resulting from crosslinking an ST2 cell in the presence of a 400-fold excess higher concentration of unlabeled OCIF than that of <sup>125</sup>I-OCIF.

~~Figure~~ Fig. 8 shows the results of SDS-PAGE in Example 9-9, wherein:

<Explanation of symbols>

Lane 1: ——— Proteins of pOBM291-transfected precipitate resulting from immuno precipitation of the protein of COS-7 cells immunoprecipitated in the absence of transfected with pOBM291 without OCIF,

Lane 2: ——— Proteins of pOBM291-transfected precipitate resulting from immuno precipitation of the protein of COS-7 cells transfected with pOBM291 with OCIF.  
———— immunoprecipitated in the presence of OCIF

~~Figure~~Fig. 9 shows the results of ~~analysis of binding capability~~experiment of  $^{125}\text{I}$ -labeled OCIF to COS-7 cells transfected with pOBM291 in ~~Example 10.10~~, wherein:

<~~Explanation of symbols~~>

Lanes 1 and 2: ~~The amount of the~~  $^{125}\text{I}$ -labeled OCIF ~~binding bound~~ to COS-7 cells transfected with pOBM291~~291~~.

Lanes 3 and 4: ~~The amount of the~~  $^{125}\text{I}$ -labeled OCIF ~~binding bound~~ to COS-7 cells transfected with pOBM291 in the presence of a 400-fold excess higher concentration of unlabeled OCIF than that of  $^{125}\text{I}$ -OCIF.

~~Figure~~Fig. 10 shows the results of ~~a crosslinking test~~experiment using OCIF- $^{125}\text{I}$ -labeled with  ~~$^{125}\text{I}$~~ OCIF in ~~Example 11.11~~, wherein:

<~~Explanation of symbols~~>

Lane 1:  $^{125}\text{I}$ -labeled OCIF,

Lane 2: sample resulting from crosslinking of  $^{125}\text{I}$ -labeled OCIF ~~crosslinked~~ with COS-7 cells transfected with pOBM291~~291~~.

Lane 3: sample resulting from crosslinking of  $^{125}\text{I}$ -labeled OCIF ~~crosslinked~~ with COS-7 cells transfected with pOBM291 in the presence of a 400-fold excess higher concentration of unlabeled OCIF than that of  $^{125}\text{I}$ -OCIF.

~~Figure~~Fig. 11 shows the results of ~~a Northern B~~lot in ~~Example 12.12~~, wherein:

<~~Explanation of symbols~~>

Lane 1: RNA ~~originating derived~~ from ST2 cells cultured ~~without addition~~in the absence of  $\text{V}$ itamin D and dexamethasone,

Lane 2: RNA ~~originating derived~~ from ST2 cells cultured ~~within~~ the addition presence of  $\text{V}$ itamin D and dexamethasone.

~~Figure~~Fig. 12 shows ~~the~~ OCIF- binding ~~capability~~ability of ~~the~~ proteins in the conditioned medium ~~at various~~when the concentration of OCIF ~~concentrations was varied~~ in ~~Example 1314-(2)~~, wherein:

<~~Explanation of symbols~~>

○: pCEP44,

●: pCEP sOBM.



~~Figure~~Fig. 13 shows the OCIF- binding ~~capability~~ability of the protein in the conditioned medium ~~at various proportions~~when the amount of the conditioned medium ~~was varied~~ in Example ~~1314~~14-(2)-, wherein:

<~~Explanation of symbols~~>

○: PCEP4

○: pCEP4,

●: PpCEP sOBM,

~~Figure~~Fig. 14 shows the results of SDS-PAGE of a fusion protein ~~consisting of~~ thioredoxin and mouse OBM expressed in ~~Escherichia~~E. coli, in Example ~~1415~~15-(2)-, wherein:

<~~Explanation of symbols~~>

Lane 1: ~~M~~molecular weight markersmarker,

Lane 2: ~~S~~soluble protein fractions originatingderived from GI724/pTrxFus,

Lane 3: ~~S~~soluble protein fractions originatingderived from GI724/pTrxOBM2525.

~~Figure~~Fig. 15 shows the OCIF- binding ~~capability~~abilities ~~at various proportions~~when the amount of the soluble protein fractions ~~were varied~~ in Example ~~1415~~15-(3)-, wherein:

<~~Explanation of symbols~~>

□: GI724/pTrxFus,

○: GI724/pTrxOBM2525.

~~Figure~~Fig. 16 shows the OCIF- binding ~~capability~~abilities of the soluble protein fractions (1%) ~~at various~~when the concentrations of OCIF ~~was varied~~ in Example ~~1415~~15-(3)-, wherein:

<~~Explanation of symbols~~>

□: GI724/pTrxFus,

○: GI724/pTrxOBM2525.

~~Figure~~Fig. 17 shows the results of inhibition of ~~the~~specific binding to OCIF- of the mouse OBM~~protein~~ obtained by expressing ~~of the~~mouse OBM cDNA of the present invention and ~~purification or purifying~~ (mouse OBM) and the ~~purified~~purified natural-OCIF-~~type~~ OCIF binding protein to OCIF, by a ~~rabbit~~anti-mouse OBM ~~rabbit~~antibody-, wherein:

<~~Explanation of symbols~~>

- 1: ~~— Purified OBM prepared by expression of the cDNA in the presence of the purified recombinant OBM treated with an antibody, OBM +  $^{125}$ I-OCIF,~~
- 2: ~~The the purified natural-type protein in the presence of the treated with an antibody +  $^{125}$ I-OCIF,~~
- 3: ~~— Mouse OBM prepared by expression of the cDNA in the absence of the purified recombinant OBM untreated with an antibody, mouse OBM +  $^{125}$ I-OCIF,~~
- 4: ~~The the purified natural-type protein in the absence of the untreated with an antibody +  $^{125}$ I-OCIF,~~
- 5: 3 + unlabeled OCIF (400-fold ~~more~~higher concentration than that of  $^{125}$ I-OCIF),
- 6: 4 + unlabeled OCIF (400-fold ~~more~~higher concentration than that of  $^{125}$ I-OCIF).

~~Figure~~Fig. 18 shows the results of SDS-PAGE of human OBM protein expressed by the cDNA of the present invention-, wherein:

<~~Explanation of symbols~~>

Lane 1: ~~M~~molecular weight markersmarker,

Lane 2: ~~— Proteins of precipitate resulting from immuno precipitation of the protein derived from COS-7 cells transfected with phOBM (an expression vector (phOBM) containing at the cDNA of the present invention), immunoprecipitated with a rabbit by an anti-OCIF rabbit polyclonal antibody in the absence of without OCIF,~~

Lane 3: ~~— Proteins of precipitate resulting from immuno precipitation of the protein derived from COS-7 cells transfected with phOBM (an expression vector (phOBM) containing at the cDNA of the present invention), immunoprecipitated with a rabbit by an anti-OCIF rabbit polyclonal antibody in the presence of with OCIF,~~

~~Figure~~Fig. 19 shows the results of ~~analysis of a~~ binding experiment of OCIF to COS-7 cells transfected with ~~phOBM~~, an expression vector (phOBM) containing ~~at the~~ cDNA of the present invention-, wherein:

<~~Explanation of symbols~~>

Lane 1: COS-7 cells transfected with phOBM ~~and the addition of~~  $^{125}$ I- OCIF-,

Lane 2: COS-7 cells transfected with phOBM ~~and the addition of~~  $^{125}$ I- OCIF-, in the presence of + a 400-fold ~~more~~higher concentration of unlabeled OCIF than that of  $^{125}$ I-OCIF.

~~Figure~~Fig. 20 shows the results of crosslinking experiment of human OBM<sub>1</sub>, ~~which is a protein encoded by a~~the cDNA of the present invention, with <sup>125</sup>I-OCIF (monomer- type)<sub>1</sub>, wherein:

<Explanation of symbols>

Lane 1: <sup>125</sup>I-OCIF<sub>1</sub>

Lane 2: ~~The crosslinked products sample resulting from crosslinking of~~ <sup>125</sup>I-OCIF with ~~the~~ proteins on the membrane of COS-7 cells transfected with phOBM<sub>1</sub>,

Lane 3: ~~The crosslinked products sample resulting from crosslinking of~~ <sup>125</sup>I-OCIF with ~~the~~ proteins on the membrane of COS-7 cells transfected with ~~pHOBM~~<sub>1</sub>, phOBM in the presence of a 400-fold ~~more~~higher concentration of unlabeled OCIF than that of <sup>125</sup>I-OCIF.

~~Figure~~Fig. 21 shows the OCIF- binding ~~capability~~ability of ~~the~~ protein (secreted~~ory-form~~hOBM<sub>1</sub> type human OBM) in the conditioned medium ~~at various~~when the concentration of OCIF concentrations was varied in Example 2324-(2)<sub>1</sub>, wherein:

<Explanation of symbols>

○: ~~Conditioned medium of 293-EBNA cells transfected with pCEP4, which does~~4 vector not containing cDNA ~~encoding secreted~~which encodes the secretory-form<sub>1</sub> type human OBM<sub>1</sub>,

●: ~~Conditioned medium of 293-EBNA cells transfected with pCEPshOBM~~<sub>1</sub>, expression vector containing cDNA which contains~~cDNA encoding secreted~~encodes the secretory-form<sub>1</sub> type human OBM<sub>1</sub>.

~~Figure~~Fig. 22 shows the OCIF- binding ~~capability~~ability of the protein (secreted~~ory-form~~type human OBM) in the conditioned medium ~~at a specific OCIF concentration~~when the amount of the conditioned medium to be added was varied while changing the amountconcentration of conditioned medium addedOCIF was kept constant, in Example 2324-(2)<sub>1</sub>, wherein:

<Explanation of symbols>

○: ~~Conditioned medium of 293-EBNA cells transfected with pCEP4, which does~~4 vector not containing cDNA ~~encoding secreted~~which encodes the secretory-form<sub>1</sub> type human OBM<sub>1</sub>,

- : Conditioned medium of 293-EBNA cells transfected with pCEPshOBM<sub>1</sub> expression vector containing cDNA which contains DNA encoding secreted encodes the secretory-form type human OBM<sub>1</sub>.

Figure Fig. 23 shows the results of SDS-PAGE of a fusion protein consisting of thioredoxin and human OBM<sub>1</sub> expressed in Escherichia *E. coli*, wherein:

<Explanation of symbols>

Lane 1: M molecular weight markers marker,

Lane 2: S soluble protein fractions originating derived from Escherichia *E. coli* GI724/pTrxFus,

Lane 3: S soluble protein fractions originating derived from Escherichia *E. coli* GI724/pTrxhOBMpTrxOBM.

Figure Fig. 24 shows the OCIF-binding capability ability of the fusion protein consisting of thioredoxin and human OBM to OCIF, when the to bind OCIF when the amount of the soluble protein fraction originating from Escherichia containing the fused protein of thioredoxin and human OBM expressed in *E. coli* including the fusion protein added was varied, in Example 2425-(3), wherein:

<Explanation of symbols>

- : S soluble protein fractions originating derived from Escherichia *E. coli* GI724/pTrxFus,

- : S soluble protein fractions originating derived from Escherichia *E. coli* GI724/pTrxshOBM.

Figure Fig. 25 shows the OCIF-binding capability ability of the fusion protein of thioredoxin and human OBM in a soluble protein fractions originating from Escherichia fraction of *E. coli* to bind OCIF in various concentrations when the concentration of OCIF was varied, in Example 2425-(3), wherein:

<Explanation of symbols>

- : S soluble protein fractions originating derived from Escherichia *E. coli* GI724/pTrxFus

- : S soluble protein fractions originating derived from Escherichia *E. coli* GI724/pTrxshOBM.

~~Figure~~Fig. 26 shows the results of quantifying measurement of human OBM and human sOBM by the sandwich ELISA method using the rabbit anti-human OBM/sOBM rabbit polyclonal antibody of the present invention-, wherein:

~~<Explanation of symbols>~~

- : Hhuman OBM<sub>1</sub>
- : Hhuman sOBM<sub>1</sub>

~~Figure~~Fig. 27 shows the results of quantifying measurement of human OBM and human sOBM by the sandwich ELISA method using the anti-human OBM/sOBM monoclonal antibodies of the present invention-, wherein:

~~<Explanation of symbols>~~

- : Hhuman OBM<sub>1</sub>
- : Hhuman sOBM<sub>1</sub>

~~Figure~~Fig. 28 shows the results of quantifying measurement of mouse OBM and mouse sOBM by the sandwich ELISA method using the anti-human OBM/sOBM monoclonal antibodies of the present invention-which, said antibody has cross-reactivity to both mouse OBM and mouse sOBM-, wherein:

~~<Explanation of symbols>~~

- : Mmouse OBM<sub>1</sub>
- : Mmouse sOBM<sub>1</sub>

~~Figure~~Fig. 29 shows the activity of the fusion protein consisting of thioredoxin and mouse OBM to stimulate promote the formation of human osteoclast-like cell formation-cells

~~Figure~~Fig. 30 shows the suppression of the vitamin D<sub>3</sub>-stimulated bone resorption by an anti-OBM/sOBM antibody of the bone resorption activity stimulated by vitamin D<sub>3</sub>-

~~Figure~~Fig. 31 shows the suppression of the anti-OBM/sOBM antibody of the bone resorption activity stimulated by prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)-stimulated bone resorption by an anti-OBM/sOBM antibody.

~~FigureFig. 32 shows the suppression by the anti-OBM/sOBM antibody of the bone-resorbing activity stimulated by parathyroid hormone (PTH)-stimulated bone resorption by an anti-OBM/sOBM antibody.~~

~~FigureFig. 33 shows the suppression by the of interleukin 1  $\alpha$  (IL-1)-stimulated bone resorption by an anti-OBM/sOBM antibody of the bone-resorbing activity stimulated by interleukin 1  $\alpha$  (IL-1).~~

## **BEST MODE FOR CARRYING OUTPRACTICING THE INVENTION**

[Examples]

~~The present invention will be describedis explained in more detail by way of examples which are given forwith reference to the purpose of illustration offollowing Examples. However, these Examples are only exemplary and shall not limit the present invention and are not limiting thereof thereto in any way of the remainder of the disclosure.~~

<[Example 1]>

### **Preparaoduction of the pProtein of the pPresent iInvention**

#### **(1) Large- sScale-cultivation Culture of ST2 eCells**

~~Mouse osteoblasticosteoblast like stromal cell line, ST22, (RIKEN CELL BANKRiken Cell Bank, RCB0224) was cultured usingwith  $\alpha$ -MEM medium containing 10% fetal-bovine fetal serum. ST2 cellsAfter cultured to confluencebecome confluent in a 225 -cm<sup>2</sup> T flask for adherent-cell-culture cells, ST2 cells were treated with trypsin and harvested, stripped from the T flask. After washing, the cells werewashed, and then transferred to five of 225 -cm<sup>2</sup> T flasks. After the addition of 60 ml of  $\alpha$ -MEM medium containing 10<sup>-8</sup> M of the active-form of vitamin D<sub>3</sub> (C<sub>1</sub>calcitriol), 10<sup>-7</sup> M dexamethasone, and 10%bovine fetal bovine serum, the resulting cells in each flask were cultured for 7-10 days in a CO<sub>2</sub> incubator for 7 to 10 days. The cultured ST2 cells were harvestedrecovered using a cell scraper and stored at -80°C until use.~~

#### **(2) Preparation of mMembrane fFraction and sSolubilization of mMembrane-bBound pProteins**

~~To the ST2 cells (volume, amount: about 12 ml) described in Example 1-(1), which were cultured using eightywith 80 of 225 -cm<sup>2</sup> T flasks, was added three times~~

thea 3-fold volume (36 ml) of 10 mM Tris-HCl hydrochloric acid buffer (pH 7.2) containing protease inhibitors (2 mM APMSFP, 2 mM EDTA, 2 mM o—phenanthroline, 1 mM leupeptin, 1 µgug/ml pepstatin A; and 100 units/ml aprotinin) were added. After these cells were vigorously agitatingagitated by use of a vortex mixer for 30 seconds using a voltex mixer, the cellsthey were allowedleft to stand on ice for 10 minutes on ice. The cells were homogenized usingUsing a homogenizer (DOUNCE TISSUE GRINDERDounce Tissue Grinder, A syringe, WHEATONWheaton SCIENTIFICScientific Co., Ltd.), these cells were crushed. The sameTo the crushed cell solution, an equal volume (48 ml) of 10 mM Tris-HCl hydrochloric acid buffer (pH 7.2) containing the above-mentioned protease inhibitors, 0.5 M sucrose, 0.1 M potassium chloride, 10 mM magnesium chloride, and 2 mM calcium chloride was added to the homogenized cells. After stirring, theThe obtained mixture was agitated and then centrifuged at 600 x g at 4°C for 10 minutes at 4°C. Through this centrifugation, thereby separating cell nuclei and non-homogenizeduncrushed cells were separated as precipitateprecipitated fractions. TheA supernatant obtained by the centrifugeafter centrifugation was further centrifuged at 150,000 x g at 4°C for 90 minutes at 4°C, to obtainand membrane fractions of the ST2 cells were obtained as precipitateprecipitated fractions. EightTo the membrane fractions, 8 ml of 10 mM Tris-HCl hydrochloric acid buffer (pH 7.2) containing the above-mentioned protease inhibitors, 150 mM of sodium chloride, and 0.1 M sucrose was added to this membrane fraction. After the addition of, and then 200 µlµl of 20% CHAPS (3-[(3-cholamidopropyl)—dimethylammonio—dimethylammonio]—1—propanesulfonate, Sigmaaigma Co.), theLtd.) was added. The mixture was stirredagitated at 4°C for 2 hours at 4°C. Theis mixture solution was then centrifuged at 150,000 x g at 4°C for 60 minutes at 4°C, to obtainand the resulting supernatant was obtained as a solubilized membrane fraction.

<[Example 2>]

### **Purification of the pProtein of the pPresent iInvention**

#### **(1) Preparation of OCIF-iImmobilized aAffinity eColumn**

After replacing iso-propanolIsopropanol in a HiTrapHITRAP® NHS-activated column (1 ml, manufactured by Pharmacia Co., Ltd.) was substituted with 1 mM hydrochloric acid, and 1 ml of 0.2 M NaHCO<sub>3</sub>/0.5 M NaCl (pH 8.3) solution (pH 8.3)

containing 13.0 mg of recombinant OCIF prepared by ~~the~~in accordance with a method  
~~of described in~~ WO 96/26217 was added to the column using a syringe (5 ml,  
~~manufactured by Terumo Corp. Corporation),~~. After the column was allowed to  
~~effect~~undergo a coupling reaction at room temperature for 30 minutes. ~~The column was~~  
~~fed with,~~ 3 ml of 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 3 ml of 0.1 M acetic  
acid/0.5 M NaCl (pH 4.0) were loaded on the column alternately three times each in  
turn total so as to inactivate excessive activated groups. Then, ~~then the solution mobile~~  
phase of the column was replacedsubstituted again with 0.5 M ethanolamine/0.5 M NaCl  
(pH 8.3). ~~After allowing and then left~~ to stand at room temperature for 1 hour.  
Thereafter, the resulting column was washed twice alternately with 0.5 M  
ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0), ~~and and~~  
then the solution mobile phase was then replacedsubstituted with 50 mM Tris/1 M  
NaCl/0.1% CAHPS buffer (pH 7.5).

(2) Purification of the pProtein of the present invention using Present Invention by an  
OCIF-immobilized Affinity Column

~~The purification~~Purification of the OCIF- binding protein was carried out at 4°C,  
unless otherwise ~~indicated~~stated. The above-mentioned OCIF-immobilized affinity  
column was equilibrated with 10 mM Tris-~~hydrochloride~~hydrochloric acid buffer (pH  
7.2) ~~to which containing~~ the protease inhibitors described in Example 1-(2), 0.15 M  
sodium chloride, and 0.5% CHAPS ~~were added.~~ AboutTo this column, about 8 ml of the  
solubilized membrane fraction described in Example 1-(2) was applied ~~to the column~~ at a  
flow rate of 0.01 ml/minute. Then, ~~the~~The column was washed with the above 10 mM  
Tris-~~hydrochloride~~hydrochloric acid buffer (pH 7.2) ~~to which containing the above-~~  
~~mentioned~~ protease inhibitors, 0.15 M sodium chloride, and 0.5% CHAPS ~~was added, for~~  
~~100 minutes~~ at a flow rate of 0.5 ml/minute in for 100 minutes. Next ~~Then,~~ the proteins  
~~adsorbed to~~ were eluted from the column ~~was eluted~~ with 0.1 M glycine-  
~~hydrochloride~~hydrochloric acid buffer (pH 3.3) containing the above protease inhibitors,  
0.2 M sodium chloride, and 0.5% CHAPS ~~for 50 minutes~~ at a flow rate of 0.1  
ml/minute in for 50 minutes. ~~In the same manner~~Similarly, the proteins adsorbed to the  
column ~~was eluted with~~ a 0.1 M sodium citrate buffer (pH 2.0) containing ~~the said~~  
protease inhibitors, 0.2 M sodium chloride, and 0.5% CHAPS ~~for 50 minutes~~ was fed to



the column at a flow rate of 0.1 ml/min for 50 minutes so as to elute proteins adsorbed to the column. The eluates ~~was collected in~~ were fractionated as 0.5 ml/fraction each. The fractions ~~Each fraction was~~ were immediately neutralized by the addition of a 2M Tris solution. The fractions ~~derived from the elution with these buffers (each fraction~~ consisting the volume of the eluate was 1.0- to 5.0 ml of eluate) eluted with the buffer were concentrated to 50- to 100 µl using Centricon 10 (manufactured by Amicon of UCENTRICON®-10 (Amersham Co. S.A., Ltd.). ~~OCIF was added to a portion of~~ each Aliquots of the concentrated fraction-fractions were subfractionated, and after addition of OCIF to the aliquots, they were immunoprecipitated with an anti-OCIF polyclonal antibody. ~~The~~ After the precipitated fractions were treated with SDS and, they were subjected to SDS-PAGE. Fractions, and then a fraction (Fr. No. Nos. 3-10) in which the showing a band of the protein with specific binding ability having an activity to specifically bind OCIF appeared were regarded was identified as the protein fractions of the present invention.

(3) Purification of the pProtein of the pPresent iInvention by gGel fFiltration

The ~~concentrated~~ OCIF- binding protein (the fractions obtain by the elution eluted with 0.1 M glycine-hydrochloridehydrochloric acid buffer (pH 3.3) and subsequently 0.1 M sodium citrate buffer (pH 2.0)) ~~prepared after purification and concentration in~~ accordance with the method described in Example 2-(2) was applied subjected to a SuperoseSUPEROSE® 12 HR10/30 column (1.0 x 30 cm, manufactured by Pharmacia Co., Ltd., 1.0 X 30 cm) ~~which was~~ equilibrated with 10 mM Tris-HCl, 0.5 M NaCl, and 0.5% CHAPS (pH 7.0) and developed with using the above equilibration buffer as a mobile phase at a flow rate of 0.5 ml/min, and each then fractions of 0.5 ml fraction waswere collected. The fractions containing the protein of the present invention (Fr. Nos. Nos. 27-32) werewas identified according to and concentrated by means of CENTRICON®-10 (Amersham Co., Ltd.) in the same methodmanner as described above. ~~Each of the fractions was concentrated using Centricon 10 (a product of~~ Amicon).

(4) Purification by rReversed pPhase hHigh pPerformance lLiquid eChromatography

~~The above mentioned~~ OCIF- binding protein purified by the above gel filtration was ~~applied~~ added to a C<sub>4</sub> column (2.1 ~~x~~ X 250 mm, Vydac, USA) ~~which was equilibrated~~ with 0.1% trifluoroacetic acid (TFA) and 30% acetonitrile. ~~The proteins bound to the column were eluted~~ Elution was carried out at a flow rate of 0.2 ml/min with linear the gradients of acetonitrile concentration of from 30% to 55% for the first 50 minutes and then of from 55% to 80% during the next for another 10 minutes at a flow rate of 0.2 ml/min. ~~Peaks~~ The peaks of eluted proteins were detected ~~by measuring optical density at 215 nm~~ mm. ~~Proteins in the different peaks were analyzed to identify the fractions containing~~ The eluted protein of each peak was fractionated, and the peak of the protein of the present invention, and was identified. Thus, a highly purified protein of the present invention was obtained.

<[Example 3]>

**SDS-PAGE of the pPurified pProtein of the pPresent iInvention**

~~The~~ First, a solubilized membrane fraction prepared from ST2 cells which were cultured in the presence or absence of the active-form of vitamin D<sub>3</sub> was ~~subjected to purification~~ purified with the OCIF-immobilized affinity column. ~~The as described~~ above, and the purified preparations ~~samples~~ were subjected to SDS-PAGE. As shown in ~~Figure~~ Fig. 1(A), it was revealed that a major protein band ~~with MW of about 30,000- to 40,000~~ was detected only in the purified ~~preparations~~ sample obtained from the ST2 cells ~~which was cultured in the presence of the active-form of vitamin D<sub>3</sub>, proving and that the~~ protein which specifically binds ~~to~~ OCIF, (i.e., the protein of the present invention) ~~can be, is~~ selectively concentrated and purified by with the OCIF-immobilized affinity column. However, ~~bands of several proteins (other than in addition to the protein of the present invention), some other bands of proteins which non-specifically were nonspecifically bound to the carriers or, spacers or the like of the OCIF-immobilized column were also detected in both of the purified preparations~~ samples. These proteins other than the protein of the present invention were removed ~~according to the above-described method~~ by gel filtration and C<sub>4</sub> reversed phase chromatography as described above. The SDS-PAGE of the obtained highly purified protein of the present invention is

shown in ~~Figure Fig.~~ 1(B). The highly purified protein of the present invention was found to be electrophoretically homogeneous, and had ~~at the~~ molecular weight ~~of thereof~~ was about 30,000- to 40,000.

<[Example 4]>

#### Examining the Binding test of OCIF to eOsteoblasts

##### (1) Preparation of $^{125}\text{I}$ -Labeled OCIF

OCIF was  ~~$^{125}\text{I}$ -labeled with  $^{125}\text{I}$~~  by the Iodogen method. ~~Specifically~~ More specifically, 20  $\mu\text{l}$  of 2.5 mg/ml Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated off at  $40^\circ\text{C}$ , ~~to obtain a tube coated with~~ so as to prepare an Iodogen-coated tube. ~~The~~ After the tube was washed three times with 400  $\mu\text{l}$  of 0.5 M sodium phosphate buffer (Na-Pi, pH 7.0). ~~Five  $\mu\text{l}$ , 5  $\mu\text{l}$~~  of 0.5 M Na-Pi (pH 7.0) was added ~~to the tube thereto~~. Immediately after ~~the addition of 1.3  $\mu\text{l}$~~  (18.5 MBq) of Na- $^{125}\text{I}$  solution (NEZ-033H20, ~~manufactured by Amersham Co.~~), Ltd., NEZ-033H20) was added to the tube, 10  $\mu\text{l}$  of 1 mg/ml rOCIF solution (monomer type or dimer type) was added ~~to the tube~~. ~~After mixing~~ The obtained solution was agitated with a vortex mixer, ~~the mixture was allowed and then left~~ to stand at room temperature for 30 seconds. The solution was transferred to a tube containing 80  $\mu\text{l}$  ~~of a solution of~~ 10 mg/ml potassium iodide ~~in and~~ 0.5 M Na-Pi solution (pH 7.0), and 5  $\mu\text{l}$  of a phosphate buffered saline solution containing 5% bovine serum albumin, and ~~stirred then~~ agitated. ~~The is mixture solution~~ was applied to a spin column (1 ml, G-25 fine, ~~manufactured by Pharmacia Co., Ltd.~~) which was equilibrated with a phosphate buffered saline solution containing 0.25% bovine serum albumin and ~~the column was~~ centrifuged at 2,000 rpm for 5 minutes ~~at 2,000 rpm~~. ~~Four hundred  $\mu\text{l}$~~  After adding 400  $\mu\text{l}$  of a phosphate buffered saline solution containing 0.25% bovine serum albumin was added to the fraction eluted from the column and ~~the mixture was stirred~~. ~~A subsequently mixed~~, 2  $\mu\text{l}$  of the aliquot was removed to measure aliquots were collected, and the radioactivity ~~using thereof~~ was measured with a gamma counter. The radiochemical purity of the thus prepared  $^{125}\text{I}$ -labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated ~~with~~ by 10% TCA. ~~The~~ Furthermore, the biological activity of as OCIF the  $^{125}\text{I}$ -labeled OCIF solution was measured ~~according to the~~ determined in accordance with a method described in WO 96/26217. ~~The~~ Moreover, the concentration

of the  $^{125}\text{I}$ -labeled OCIF was measured by the ELISA according to the following procedure manner.

(2) Measurement of the concentration of  $^{125}\text{I}$ -labeled OCIF by ELISA

~~One hundred~~ 100  $\mu\text{l}$  of 50 mM  $\text{NaHCO}_3$  (pH 9.6) in which the 2  $\mu\text{g}/\text{ml}$  of anti-OCIF rabbit polyclonal antibody described in WO 96/26217 was dissolved to a concentration of 2  $\mu\text{g}/\text{ml}$  was added to each well of a 96-well immunoplate (MaxiSorp<sup>TM</sup>, a product of Nunc Co.). The plate was allowed, Ltd.) and left to stand at 4°C overnight at 4°C. After removing the this solution by was suction removed, 300  $\mu\text{l}$  of Block-Ace<sup>TM</sup> BLOCKACE (Snow Brand Milk Products Co., Ltd.)/phosphate buffered saline (25/75)-solution (25/75) was added to each well. The plate was and then allowed left to stand for two hours at room temperature for 2 hours. After removing the this solution by suction, the was removed, each wells were was washed three times with phosphate buffered saline solution (P-PBS) containing 0.01% Polysorbate 80 (P-PBS)-polysorbate 80. Next Thereafter, 300  $\mu\text{l}$  of Block-Ace<sup>TM</sup> BLOCKACE/phosphate buffered saline (25/75)-solution to which (25/75) containing  $^{125}\text{I}$ -labeled OCIF sample or the standard OCIF preparation was mixed, was added to each well. The plate was then allowed and left to stand for two hours at room temperature for 2 hours. After removing the this solution by was suction removed, each well was washed six times with 200  $\mu\text{l}$  of P-PBS. One hundred  $\mu\text{l}$  Then, 100  $\mu\text{l}$  of Block-Ace<sup>TM</sup> BLOCKACE (Snow Brand Milk Products Co., Ltd.)/phosphate buffered saline (25/75)-solution (25/75) containing peroxidase -labeled rabbit anti-OCIF rabbit polyclonal antibody was added to each well. The plate was allowed and left to stand for two hours at room temperature for 2 hours. After removing the this solution by was suction removed, the each wells were was washed six times with 200  $\mu\text{l}$  of P-PBS. Then, 100  $\mu\text{l}$  Then, 100  $\mu\text{l}$  of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well. After incubating and then left to stand at room temperature for 2- to 3 minutes. Thereafter, 100  $\mu\text{l}$  of stopping solution (Stopping Reagent, (Scytek Co., Ltd.) was added to each well. Absorbance The absorbance of each well at 490 nm was measured at 490 nm using with a microplate reader. The concentration of the  $^{125}\text{I}$ -labeled OCIF was determined calculated from a calibration curve prepared made by using the standard preparation of OCIF.

(3) Examining the Binding test of OCIF to Osteoblasts or spleenPancreas eCells

Mouse ~~osteoblastic~~osteoblast-like stromal cell line, ST22, or ~~spleen~~mouse ~~pancreas~~ cells were suspended in  $\alpha$ -MEM medium containing 10% bovine fetal ~~bovine~~ serum (FBS), either with or without  $10^{-8}$  M of the active-form of vitamin D<sub>3</sub> (Ccalcitriol) and  $10^{-7}$  M dexamethasone, ~~to~~ at a concentration of  $4 \times 10^4$  cells/ml (~~ST~~and 2 cells) or  $2 \times 10^6$  cells/ml (~~spleen cells~~), respectively. ~~Each cell suspension~~ 1 ml of this medium was ~~innoculated~~seeded ~~into~~ a 24- well micro-platemicroplate. ~~The~~After the cells were cultured ~~for 4 days in~~ in a CO<sub>22</sub> incubator. ~~After washing the cells for 4 days and washed~~ with  $\alpha$ -MEM medium, 200  $\mu$ l of medium for the binding ~~test~~experiment ( $\alpha$ -MEM ~~to~~ which medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, and 0.2% NaN<sub>3</sub> ~~were supplemented~~), further containing 20 ng/ml of the above-described <sup>125</sup>I-labeled OCIF (monomer ~~form~~type or dimer ~~form~~type), was added to each well. ~~To the wells for the measurement of non-specific binding~~Furthermore, 200  $\mu$ l of the medium for the binding ~~test~~experiment containing 8  $\mu$ g/ml ~~of~~ rOCIF (400-times-fold higher concentration) ~~in addition to 20 ng/ml of~~ <sup>125</sup>I-labeled OCIF was added. ~~The to other wells which were subjected to measurements of nonspecific binding.~~ After the cells were cultured ~~for one hour in~~ in a CO<sub>22</sub> incubator ~~and for 1 hour, they were washed 3~~three times with 1 ml of a-phosphate buffered saline solution. ~~In this procedure~~Since pancreas cells are floating cells, spleen cells in each well were washed by centrifuging in the 24-well plate in each washing step, because the spleen cells were non-adherent with centrifugation. After washing, 500  $\mu$ l of 0.1 N NaOH solution was added to each well and ~~the plate was allowed~~left to stand for 10 minutes at room temperature ~~to dissolve for 10 minutes.~~ Thereby, the cells. ~~The were washed, and the amount of RI in each well bound to the cells~~ was measured by with a gamma counter.

~~As shown in Figure 2, The~~ <sup>125</sup>I-labeled OCIF did not bind ~~to the cultured spleenpancreas cells, but specifically bound only to the osteoblasticosteoblast like stromal cells which were cultured in the presence of the active-form of vitamin D<sub>3</sub>.~~ The results indicated Thereby, it was revealed that the protein of the present invention ~~is~~was a membrane -bound protein induced ~~by~~on the cell surface of osteoblast like stromal cells with the active-form of vitamin D<sub>3</sub> and dexamethasone on osteoblastic stromal cells.

<[Example 5]>

## **Biological ~~a~~Activity of the ~~p~~Protein of the ~~p~~Present ~~i~~Invention**

### **(1) ~~— Osteoclasts formation supported by osteoblastic stromal cells~~**

#### **(1) Ability of Osteoblast Like Stromal Cells to Support Osteoclast Formation**

~~The osteoclasts~~ability of osteoblasts to support osteoclast formation supporting  
~~capability of osteoblastic stromal cells was evaluated~~examined by measuring tartaric acid  
resistant acid phosphatase activity (TRAP activity) of the formed osteoclasts.

~~Specifically~~More specifically, spleen mouse osteoblast-like stromal cell line, ST2, (5 X  
10<sup>3</sup> cells/100  $\mu$ l/well) (2  $\times$  10<sup>5</sup> cells/100  $\mu$ l/well) and pancreas cells derived from a  
ddy mouse (8- to 12 weeks old) and mouse osteoblastic stromal cells ST2 (5  $\times$  10<sup>3</sup>  
cells/100  $\mu$ l/well)-were suspended in  $\alpha$ -MEM to which 10<sup>-8</sup>-medium containing 10%  
bovine fetal serum, 10<sup>-8</sup> M of the active-form vitamin D<sub>3</sub>, 10<sup>-7</sup> of vitamin D<sub>3</sub> and 10<sup>-7</sup> M  
dexamethasone, and 10% fetal bovine serum were added~~seeded~~in a 96-well plate.  
~~The~~After the cells were inoculated into 96-well plates and cultured in a CO<sub>2</sub> incubator  
for one week in a CO<sub>2</sub> incubator. After washing, each well was washed with phosphate  
buffered saline solution. Then, 100  $\mu$ l of ethanol/acetone (1:1) was further added to  
each the well, wells and the cells were immobilized~~fixed~~at room temperature for one  
minute. After immobilization~~Then, 100  $\mu$ l of 50 mM citrate~~citric acid buffer (pH 4.5)  
containing 5.5 mM p-nitrophenol phosphate and 10 mM sodium tartarate was added to  
each well. After and then allowed to react at room temperature for 15 minutes. After  
of the reaction at room temperature, 0.1 N NaOH solution was added to each well, and the  
absorbance at 405 nm was measured using with a microplate reader. The~~Fig. 3 shows the~~  
results of osteoclasts formation by examining the abilities of ST2 cells with a to support  
osteoclast formation, wherein the passage number of about said cells were around 10's or  
around 40's (after purchasing the cells purchased from RIKEN CELL BANK are shown  
in Figure 3-Riken Cell Bank). The~~From these results indicate, it was revealed that the~~  
ST2 cells with of a higher passage number exhibit more potent~~had a high~~ability to  
support osteoclasts- formation.

### **(2) ~~— Time course change of expression of the protein of the present invention on~~**

~~membrane of osteoblastic stromal cells in a culture system which include active-~~

~~form vitamin D<sub>3</sub> and dexamethasone and time course change~~

(2) Changes with the Passage of Time in Expression of osteoclasts formation the Protein of the Present Invention on the Membrane of Osteoblast Like Stromal Cells Cultured in the co-culture system the Presence of the Active-Form of Vitamin D<sub>3</sub> and Dexamethasone and Those in Osteoclast Formation in a Co-Culture System

~~In the same manner as in Example 4(3), osteoblastic~~Osteoblast-like stromal cell line, ST22, was cultured ~~for 7 days~~ in the presence of the active-form of vitamin D<sub>3</sub> and dexamethasone for 7 days in the same manner as in Example 4-(3). ~~The OCIF-binding test experiment was carried out~~conducted using <sup>125</sup>I-labeled OCIF (monomer type) as described in ~~the experiment in~~ Example 4-(1). ~~Non-specific~~Nonspecific binding was measured by competing the <sup>125</sup>I-labeled OCIF binding to ST2 cells with a 400-fold higher concentration of unlabeled OCIF in binding to ST2 cells. As a result, ~~it was confirmed that the amount of specific binding of the <sup>125</sup>I-labeled OCIF increase in accordance with increase in culture period in~~was increased, due to the presence of active-form of vitamin D<sub>3</sub> and dexamethasone, with an increase in culturing days. Specifically That is, as shown in Figures 4 and 5, the protein of the present invention was expressed on the cell surface of ST2 cells ~~by due to the active-form of vitamin D<sub>3</sub> in accordance with an increase in cultureing period days, and the its~~ expression reached a maximum on the fourth day of culture. On the other hand, osteoclast-like cells ~~are were~~ formed ~~by co-culturing after co-culture of~~ mouse spleen cells and ST2 cells in the presence of the active-form of vitamin D<sub>3-3</sub>. TRAP (a marker enzyme of osteoclasts)—positive mononuclear pre-osteoclast-like cells are were formed on the third or fourth day of ~~the culture. More, and further,~~ differentiated and matured TRAP-positive multinuclear cells are were formed on the fifth ~~to or~~ sixth day of ~~the culture. A good correlation between~~ It was found that change with the passage of time course of the in expression of the protein of the present invention and ~~osteoclasts in osteoclast formation was thus demonstrated~~corresponded well with each other.

(3) — Inhibition of osteoclasts formation by OCIF treatment for different period during the co-culture

(3) Effect of Inhibiting Osteoclast Formation When OCIF Was Treated Only During a Restricted Period of Co-Culture

To ~~make it clear~~ further clarify that the protein of the present invention ~~is~~ was a factor involved in ~~the osteoclast osteoclasts~~ osteoclasts formation, ~~the cells cultured during various periods (two days each, except for the fifth day) were treated with 100 mgng/ml of OCIF for different culture periods during the six in the above 6-day co-culture period described in the above mentioned Example 5(2) (two consecutive days in the six day period, except for the 5th day for which a one day period was applied(2)).~~ As a result, ~~as shown in Figure~~ Fig. 6, ~~in the case where OCIF treatment at was added during the 48-th to 96-hours after start of th hr (as counted from the beginning of culture at which expression of), when the protein of the present invention was expressed at highest level on ST2 cells is maximal was found to be most effective for inhibiting, osteoclast formation of osteoclasts was inhibited most effectively. Specifically That is, it was confirmed revealed that OCIF controls inhibited osteoclast formation by binding to ST2 cells via the protein of the present invention.~~

~~Based on the results of From~~ the above experiments results, it became clear that the protein of the present invention ~~was confirmed to be induced on cell membrane of osteoblasti~~ the membranes of osteoblast-like stromal cells by with the active-form of vitamin D<sub>3</sub> and dexamethasone, and to exhibit a had the biological activity to support or accelerate(effect) of a factor which supports and promotes differentiation or and maturation of osteoclasts.

<[Example 6]>

**Crosslinking test for Experiment of <sup>125</sup>I-labeled OCIF and to the pProtein of the pPresent iInvention**

To further identify the presence of the protein of the present invention ~~more clearly,~~ <sup>125</sup>I-labeled OCIF was allowed to crosslink with the protein of the present invention was crosslinked with <sup>125</sup>I-labeled OCIF. ~~Mouse osteoblastic stromal~~ As in Example 4-(3), mouse osteoblast like cell line, ST22, was cultured for four days in the presence or absence of the active-form of vitamin D<sub>3</sub> and dexamethasone in the same manner as described in Example for 4(3) days. After washing the cells were washed with 1 ml of phosphate buffered saline solution, 200 µl of medium for binding



~~test experiment (α-MEM to which medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.2% NaN<sub>3</sub> and 100 μg/ml heparin were added), further containing 25 ng/ml of the above <sup>125</sup>I-labeled OCIF (monomer type) or 40 ng/ml of <sup>125</sup>I-labeled OCIF-CDD1 which was added. <sup>125</sup>I-labeled OCIF-CDD1 was obtained by expressing the protein of Sequence described as SEQ ID No. NO: 76 (in WO 96/26217) in with animal cells, was added. The and labeling in accordance with the above-mentioned culture method. Furthermore, the medium for the binding test was further supplemented with experiment, containing a 400-fold higher concentration of OCIF and, was added to the other well well and was subjected to assess non-specific experiment for nonspecific binding. After culturing for one hour the cells were cultured in a CO<sub>2</sub> incubator for 1 hour, each well was they were washed three times with 1 ml of phosphate buffered saline solution containing 100 μg/ml of heparin. Five Then, hundred 500 μl of phosphate buffered saline containing solution in which 100 μg/ml of crosslinking agent, DSS (Disuccinimidyl suberate, Pierce Co.), Ltd.) was dissolved was added to each well thereto, and the plate was kept allowed to react at 0°C for 10 minutes at 0°C. The After the cells in these wells were washed twice with 1 ml of phosphate buffered saline at solution cooled to 0°C. One, hundred 100 μl of 20 mM Hepes buffer containing 1% Triton X-100, 10 μM pepstatin, 10 μM leupeptin, 2 mM PMSF (phenylmethylsulfonyl fluoride), 10 μM pepstatin, 10 μM leupeptin, 10 μM antipain, and 2 mM EDTA, was then added to each well. The plate was allowed, and left to stand for 30 minutes at room temperature to dissolve for 30 minute so as to lyse the cells. Fifteen After 15 μl of these samples were treated with SDS under non-reducing conditions according to conventional method and subjected to in accordance with a commonly used method, they were run on a SDS-polyacrylamide gel-electrophoresis (4-gel (with a gradient of 4 to 20% polyacrylamide gradient, manufactured by Daiichi Chemical Pure Chemicals Co., Ltd.). After electrophoresis, the gels were was dried and exposed to BioMax BIOMAX® MS film (manufactured by Kodak Co., Ltd.) for 24 hours at -80°C using BioMax BIOMAX® MS intensifying screens (manufactured by amplifying screen (Kodak) Co., Ltd.) at -80°C for 24 hours. After exposure, the film was The exposed films were developed by in conventional accordance with a commonly used method. A band of crosslinking product with When the <sup>125</sup>I-labeled OCIF (monomer~~

type, 60 kDa) was used, a crosslinked protein having a molecular weight of about 90,000- to 110,000 was detected. On the other hand, when the  $^{125}\text{I}$ -labeled OCIF (monomer type, 60 kDa) was used. When the  $^{125}\text{I}$ -labeled OCIF-CDD1 (31 kDa) was used, a band of crosslinking product crosslinked protein of about 70- to 80 kDa (78 kDa on average, 78 kDa) was detected as shown in Figure Fig. 7.

<[Example 7]>

#### Scatchard Plot Analysis of the pProtein of the pPresent iInvention eExpressed on ST-cells by Seatchard Plot2 Cells

The above-mentioned  $^{125}\text{I}$ -labeled OCIF (monomer type) was added to a concentration of 1,000 pM to the culture medium Medium for the binding test experiment ( $\alpha$ -MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, and 0.2%  $\text{NaN}_3$ ) and the culture medium further containing 1,000 pM of the above  $^{125}\text{I}$ -labeled OCIF (monomer type) was serially prepared and diluted stepwise at a dilution rate to of 1/2 with the culture medium not containing  $^{125}\text{I}$ -labeled OCIF for the binding experiment. Solutions Furthermore, another medium for measuring determining non-specific non-specific binding were was prepared by further adding a 400-fold higher concentration of unlabeled monomer-form type OCIF to these solutions the above medium. Two hundred  $\mu\text{L}$  of these prepared solutions were added to the above-mentioned wells with of the above ST2 cells (about 10<sup>th</sup> passage), cultured for 4 days (passage number, about 10) in the presence of  $10^{-8}$  M of the active-form of vitamin D<sub>3</sub> (Calcitriol) and  $10^{-7}$  M dexamethasone, to assess and binding of the  $^{125}\text{I}$ -labeled OCIF was tested in the same method manner as described in Example 4-(3). The obtained results were subjected to Scatchard Plot analysis to determine the plotted in accordance with a common method, and dissociation constants of OCIF and the OCIF- binding protein, and the number (site) of OCIF of the OCIF binding protein (site) per a one ST2 cell were determined. As a result, the dissociation constants of OCIF and the protein of the present invention was found to be were 280 pM, and the number of the site of OCIF-binding protein (site) per a one ST2 cell was approximately about 33,000/cell. Based on the finding in Example 5(1) that osteoclasts formation supported by the Furthermore, a cultured ST2 cells with a passage number about number of around 40 was more extensive 's had higher ability to support osteoclast formation than that with a passage number

~~about 10, the number (the site) of around 10's as shown in Example 5-(1), so that the~~  
number of sites of the protein of the present invention expressed on the ST2 cell with a  
passage number ~~about~~of around 40's was ~~assessed~~measured. ~~The~~As a result, the number  
~~(of site)~~ was 58,000/cell ~~and~~which, was clearly ~~larger~~greater than ~~that on~~ the ST2 cells  
with a ~~passage number about 10, indicating of around 10's~~. It was ~~revealed~~ that the  
amount of the expression of the protein of the present invention ~~expressed on~~associated  
with the degree of the ability of ST2 cells is related to their potency cell to support  
osteoclasts- formation. ~~The is results- finding indicateds~~ that the protein of the present  
invention is a factor ~~that to supports or induces and promote~~ differentiation ~~or and~~  
maturation of osteoclasts.

<[Example 8]>

#### Cloning of OBMeDNAOBM cDNA

##### (1) Extraction of RNA from mMouse ST2 eCells

Mouse ~~osteoblastic~~osteoblast-like stromal cell line, ST2~~2~~, (~~RIKEN CELL~~  
~~BANK~~Riken Cell Bank, RCB0224) was ~~cultured in~~with  $\alpha$ -MEM medium (Gibco BRL  
Co., Ltd.) containing 10% fetal-bovine fetal serum. ~~ST2 cells~~After cultured to cells  
become confluent in a 225 -cm<sup>2</sup> T- flasks for adherent cell culture, ST2 cells were treated  
with trypsin ~~to harvest the cells~~, stripped from the T- flask. ~~The cells were~~, washed, and  
transferred to five 225 -cm<sup>2</sup> T- flasks. ~~Sixty~~After adding 60 ml of  $\alpha$ -MEM medium  
containing 10<sup>-8</sup> M of the active-form of vitamin D<sub>3</sub> (~~C~~calcitriol, Wako Pure Chemicals  
Co.Industries, Ltd.), 10<sup>-7</sup> M dexamethasone; and 10% fetal-bovine fetal serum ~~was added~~  
~~to each flask and thereto~~, the cells were cultured ~~for 5 days~~ in a CO<sub>2</sub> incubator for 5 days.  
Total RNA was extracted from the cultured ST2 cells using ISOGEN (Wako Pure  
Chemicals Co.Industries, Ltd.). Poly A<sup>+</sup> RNA was prepared from about 600  $\mu$ g of the  
total RNA using an Oligo (dT)-cellulose column (5'-3' Prime Co., Ltd.). About 8  $\mu$ g  
of ~~P~~poly A<sup>+</sup> RNA was obtained.

##### (2) Construction of eExpression lLibrary

Double- stranded cDNAs ~~was~~ were synthesized from 2  $\mu$ g of ~~polyA~~the poly A<sup>+</sup>  
RNA obtained in Example 8-(1) ~~using a~~with Great Lengths cDNA Synthesis kit  
(Clontech Co., Ltd.) ~~according to the instruction in the~~accordance with a manual thereof.  
~~Specifically~~More specifically, 2  $\mu$ g of ~~polyA~~the poly A<sup>+</sup> RNA and an Oligo (dT)<sub>25</sub> (dN)

primer were mixed ~~and together~~, distilled water was added ~~to the mixture to make~~ thereto so that the final volume ~~to was~~ 6.25  $\mu$ l. ~~After incubation, and the mixture was incubated~~ at 70°C for about 3 minutes at 70°C, the mixture was and then cooled ~~on~~ in ice for 2 minutes. ~~To the mixture were added~~ Then, 2.2  $\mu$ l of distilled water, 2.5  $\mu$ l of 5X First-strand buffer, 0.25  $\mu$ l of 100 mM DTT (dithiothreitol), 0.5  $\mu$ l of PRIME RNase inhibitor (1 U/ml) (5'-3' Prime Co., Ltd.), 0.5  $\mu$ l of [ $\alpha$ -<sup>32</sup>P] dCTP (Amersham Co., Ltd., 3,000 Ci/mmol), 2  $\mu$ Ci/ $\mu$ l) which was diluted 5-fold with distilled water to make 2  $\mu$ Ci/ $\mu$ l to be one fifth concentration, 0.65  $\mu$ l of dNTP (20 mM each), and 1.25  $\mu$ l (250 units) of MMLV (RNaseH<sup>-</sup>) reverse transcriptase were added thereto, respectively. The mixture Thus obtained solution was incubated at 42°C for 90 minutes at 42°C. Then, followed by the further addition of 62.25  $\mu$ l of distilled water, 20  $\mu$ l of 5X second-strand buffer, 0.75  $\mu$ l of dNTP (20 mM each), and 5  $\mu$ l of Second-strand enzyme cocktail. The resulting mixture were added thereto, respectively. Thus obtained solution was maintained incubated at 16°C for two 2 hours. Then, 7.5 units of T4 DNA polymerase was added to this reaction mixture. After incubation thereto, and further incubated at 16°C for another 30 minutes. Thereafter, the reaction was terminated by the addition of 5  $\mu$ l of 0.2 M EDTA. After was added to terminate the reaction, and after a phenol-chloroform treatment, the product was precipitated with ethanol precipitation was carried out. An EcoRI-SalI-NotI linker (Clontech Co., Ltd.) was attached added to the ends of the resultant double-stranded cDNA. Then, the ends were phosphorylated and the product was applied on then phosphorylated at its end. Using a column for size fractionation column to obtain cDNA with a length more, cDNAs of not smaller than 500 bp. DNA was were separated, and ethanol-precipitated. The precipitated with ethanol, dissolved DNAs were reconstituted in water and ligated inserted to into pcDL-SR  $\alpha$ -296 (Molecular and Cellular Biology, Vol. 8, pp. 466- to 472, 1988) which had been out (Takara Shuzo Co., Ltd.) previously cleaved with a restriction enzyme, EcoRI (Takara Shuzo Co.), and subsequently treated with CIAP (calf intestine alkaline phosphatase, Takara Shuzo Co., Ltd.).

(3) Screening of eExpression Library by means of in Which the bBinding to OCIF Was Used as an Index

~~An escherichia~~E. coli ~~strail~~, XL2 Blue MRF' (Toyobo Co., Ltd.); was transformed ~~usingwith~~ the DNA ~~produced~~obtained in Example 8-(2), and ~~cultured~~allowed to grow on a ~~L-Carbenisilin agar~~ Carbenicillin Agar Medium (1% trypton, 0.5% yeast extract, 1% NaCl, 60  $\mu\text{g/ml}$  ~~carbenisilin~~, carbenicillin and 1.5% agar) prepared in a 24-well plastic ~~plates~~, plate for cell culture so that the cells was grown to ~~produce~~ about 100 colonies per well. ~~Transformants~~The transformants in each well were suspended in 3 ml of Terrific Broth ampicillin ~~culture-medium~~ (1.2% trypton, 2.4% yeast extract, 0.4% glycerol, 0.017 M  $\text{KH}_2\text{PO}_4$ , 0.072 M  $\text{K}_2\text{HPO}_4$ , 100  $\mu\text{g/ml}$  ampicillin), and cultured with shaking at 37°C ~~overnight-with-shaking~~. Cells ~~were~~The E. coli was collected by centrifugation ~~to prepare,~~ and plasmid DNA ~~using a QIAwell~~DNAs were prepared therefrom with QIAWELL® kit (QIAGEN Co., Ltd.). The DNA ~~concentration~~content was determined by ~~measuring~~detecting absorbance at 260 nm. ~~DNA,~~ and the DNAs was concentrated by ~~precipitating-with-ethanol~~ precipitation and dissolved in distilled water ~~to aso that the~~ concentration ~~of~~was 200 ng/ $\mu\text{l}$ . ~~Five-hundred~~Thus, 500 DNA pools; each ~~of which was~~ obtained~~derived~~ from about 100 colonies were prepared and ~~were~~ used for transfection ~~into~~of COS-7 cells (~~RIKEN CELL BANK~~Riken Cell Bank, RCB0539). COS- 7 cells were seeded into ~~DMEM~~ containing 10% fetal bovine serum in each well ~~of a~~ 24-well ~~plates~~ at a cell density of plate so as to achieve  $8 \times 10^4$  cells/well and cultured ~~overnight~~ at 37°C in a  $\text{CO}_2$  incubator at 37°C overnight by use of DMEM medium containing 10% bovine fetal serum. ~~Next~~On the following day, the ~~culture-medium~~ was removed, and the cells were then washed with serum-free DMEM culture-medium. ~~The above-described~~In accordance with a protocol attached to lipofectamine (Gibco Co., Ltd.) which was a reagent for transfection, the plasmid DNA ~~which was previously diluted with an~~ OPTI-MEM-culture® medium (Gibco BRL Co., Ltd.) and lipofectamine were mixed with Lipofectamine ~~(a transfection reagent~~together, manufactured by Gibco BRL Co.) according to the protocol supplied with Lipofectamine. ~~After~~and after 15-minute minutes~~inculation~~, the mixture was added to the cells in each well. The amounts of LipofectamineDNA and DNAlipofectamine used were, respectively, 1  $\mu\text{g}$  and 4  $\mu\text{l}$  per well, respectively. After 5-hour hours~~incubation~~, the ~~culture-medium~~ was removed, and

1 ml of DMEM culture-medium (Gibco BRL Co., Ltd.) containing 10% fetal-bovine fetal serum was added to each well. The plates were incubated for 2-3 days at 37°C and cultured in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) at 37°C for 2 to 3 days. The COS-7 cells transfected obtained after transfection and cultured subsequent culture for 2- to 3 days in this manner were washed with a serum-free DMEM culture medium. Then, 200 µl of a culture-medium for the binding assay experiment (serum-free DMEM culture-medium containing 0.2% calf-bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin, and 0.02% NaN<sub>3</sub>) with further containing 20 ng/ml of <sup>125</sup>I-labeled OCIF was added thereto was added to each well. After culturing for one hour at 37°C Cells were cultured in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>), the cells were at 37°C for 1 hour and washed twice with 500 µl of a phosphate buffered saline solution containing 0.1 mg/ml heparin. After washing, 500 µl of 0.1 N NaOH solution was added to each well. The plates were allowed thereto, and then left to stand for 10 minutes at room temperature for 10 minutes so as to lyse the cells. The amount of <sup>125</sup>I in each well was measured using with a gamma counter (Packard Co., Ltd.). One DNA pool containing cDNA encoding the protein which specifically binds to OCIF was found by After screening a total of the 500 pools. The in total, a DNA pool containing the a cDNA encoding a protein that could specifically bind OCIF was further divided isolated. Furthermore, the DNA pools containing the cDNA of the present invention were subfractionated, and then employed to repeat the above-described transfection and screening operations were repeated to isolate the. Thereafter, a cDNA which encodes the encoding a protein which binds could to bind OCIF was isolated. The A plasmid containing this cDNA was named referenced pOBM291. The Escherichia coli containing this plasmid was deposited with at The National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Biotechnology Laboratory, as pOBM291 on May 23, 1997 under with the deposition No. number of FERM BP-5953. The methods of 5953 on May 23, 1997. Methods for <sup>125</sup>I-labeling of OCIF with <sup>125</sup>I and quantitative analysis of determining (the concentration of) <sup>125</sup>I-labeled OCIF by ELISA are shown as below follows. Labeling of OCIF was <sup>125</sup>I-labeled in accordance with <sup>125</sup>I was carried out according to the Iodogen method. Twenty 20 µl of 252.5 mg/ml Iodogen-chloroform solution was added transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated by heating at 40°C, so as to

prepare an Iodogen-coated tube. ~~The~~After the tube was washed three times with 400  $\mu\text{l}$  of 0.5 M sodium phosphate buffer (Na-Pi, pH 7.0), ~~and 50.5  $\mu\text{l}$  of 0.5 M Na-Pi (with a pH of 7.0) was added thereto.~~ Immediately after ~~the addition of 1.3  $\mu\text{l}$  (18.5 MBq) of Na-<sup>125</sup>I solution (NEZ-033H20, Amersham Co.), Ltd., NEZ-033H20) was added thereto,~~ 10  $\mu\text{l}$  of 1 mg/ml ~~OCIF~~OCIF solution (monomer type or dimer type) was added to the tube. ~~After mixing the contents~~The resulting solution was agitated with a vortex mixer, ~~the tube was allowed and then left~~ to stand at room temperature for 30 seconds. This solution ~~in the tube~~ was transferred to a tube ~~in which 80  $\mu\text{l}$  of 10 mg/ml potassium iodide, 80  $\mu\text{l}$  of 0.5 M Na-Pi solution (pH 7.0) and 5  $\mu\text{l}$  of a phosphate buffered saline solution containing 5% bovine serum albumin (BSA-PBS) were~~was previously added and then agitated. ~~After stirring, the mixture~~This solution was applied to a spin column (1 ml, G-25 fine, ~~manufactured by Pharmacia Co., Ltd.~~) equilibrated with BSA-PBS; and ~~the column was centrifuging for 5 minutes~~centrifuged at 2000  $\pm$  200 rpm for 5 minutes. ~~Four~~After ~~hundred~~400  $\mu\text{l}$  of BSA-PBS was added to the fraction elutedan eluate from the column. ~~After stirring and mixed, 2  $\mu\text{l}$  of an aliquot of this solution was sampled to measure the~~subfractionated and its radioactivity ~~by~~was measured with a gamma counter. The radiochemical purity of the <sup>125</sup>I-labeled ~~OCIF~~ solution thus prepared <sup>125</sup>I-labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated ~~by~~with 10% TCA. ~~The~~Furthermore, the biological activity as OCIF of the <sup>125</sup>I-labeled OCIF solution was ~~measured according to the~~determined in accordance with a method ~~of~~described in WO 96/26217. ~~The~~Moreover, the concentration of the <sup>125</sup>I-labeled OCIF was ~~determined~~measured by the ELISA as followsin the following manner. SpecificallyThat is, 100  $\mu\text{l}$  of 50 mM NaHCO<sub>3</sub> (pH 9.6) in which the 2  $\mu\text{g/ml}$  of anti-OCIF rabbit polyclonal antibody described in WO 96/26217 was dissolved to a concentration of 2  $\mu\text{g/ml}$  was added to each well of a 96-well immune plateimmunoplate (MaxiSorp™, a product of Nunc Co.). ~~The plate was allowed, Ltd., MaxiSorp) and left to stand over night at 4°C overnight.~~ After removing thethis solution ~~by~~was suctionremoved, 300  $\pm$  200  $\mu\text{l}$  of Block-Ace™a combined solution of of BLOCKACE (Snow Brand Milk Products Co., Ltd.) and phosphate buffered saline solution (mixing ratio = 25:75); (B-PBSBPB) was added to each well. ~~The plate was and then allowed~~left to stand for two hours at room temperature for 2 hours. After removing thethis solution

~~by~~ ~~was~~ ~~suction~~ ~~removed~~, ~~the~~ ~~each~~ ~~well~~ ~~s~~ ~~were~~ ~~was~~ washed three times with phosphate buffered saline solution (P-PBS) containing 0.01% Polysorbate 80 (~~P-PBS~~)-80. Next ~~Thereafter~~, 100  $\mu$ l of B-PBS containing a  $^{125}$ I-labeled OCIF or standard OCIF was added to each well. ~~The plate was then allowed thereto and left to stand for two hours at room temperature for 2 hours.~~ After ~~removing the~~ ~~this~~ ~~solution~~ ~~by~~ ~~was~~ ~~suction~~ ~~removed~~, each well was washed six times with 200  $\mu$ l of P-PBS. ~~One hundred  $\mu$ l of~~ ~~Then~~, a peroxidase-labeled ~~rabbit~~-anti-OCIF ~~rabbit~~ polyclonal antibody was diluted with B-PBS and 100  $\mu$ l of the diluted solution was added to each well. ~~The plate was allowed, and then left to stand for two hours at room temperature for 2 hours.~~ After ~~removing the~~ ~~this~~ ~~solution~~ ~~by~~ ~~was~~ ~~suction~~ ~~removed~~, ~~the~~ ~~each~~ ~~well~~ ~~s~~ ~~were~~ ~~was~~ washed six times with 200  $\mu$ l of P-PBS. Then, 100  $\mu$ l of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well. ~~After incubating the plate and then left to stand at room temperature for 2- to 3 minutes.~~ ~~Thereafter~~, 100  $\mu$ l of stopping solution (Stopping Reagent; (Scytek Co., Ltd.) was added ~~to~~ ~~thereto~~. The absorbance of each well. Absorbance at 450 nm ~~of each well was measured using~~ ~~with~~ a microplate reader. The concentration of ~~the~~  $^{125}$ I-labeled OCIF was determined ~~based on~~ ~~from~~ a calibration curve ~~drawn~~ ~~made~~ using ~~the~~ ~~standard~~ ~~preparation~~ ~~of~~ OCIF.

(4) Determination of the ~~n~~Nucleotide sSequence of the cDNA encoding Which Eccodes the ~~entire~~ amino acid sequence Full Length Amino Acid Sequence of OBM

The nucleotide sequence of ~~the~~ OBM cDNA obtained in ~~the~~ Example 8-(3) was determined ~~using a~~ ~~with~~ Taq ~~Dye~~ ~~Deoxy~~ ~~Dye~~ ~~Deoxy~~ Terminator Cycle Sequencing kit (a ~~product of~~ Perkin Elmer Co., Ltd.). ~~Specifically~~ ~~That is~~, using pOBM291 as a template, the nucleotide sequence of the inserted fragment was directly determined ~~using pOBM291 as a template.~~ ~~Fragments with a length of~~ ~~Furthermore~~, about 1.0 kb and ~~about 0.7 kb which were~~ ~~fragments~~ obtained by ~~digesting~~ ~~cleaving~~ pOBM291 with a restriction enzyme, EcoRI, were inserted into ~~the~~ EcoRI site of plasmid pUC19 (Takara Shuzo Co., Ltd.) and sequenced, respectively. ~~The nucleotide sequences of these fragments were also determined.~~ The following primers were used: ~~A~~ primer SRR2 which was used to ~~determine nucleotide sequences of~~ ~~for sequencing the~~ DNA fragments inserted ~~into~~ ~~in~~ pcDL-SR ~~at~~  $\alpha$ 296, primers M13PrimerM3 and M13PrimerRV (both manufactured by Takara Shuzo Co., Ltd.) ~~which were used to determine~~ ~~for sequencing the~~ ~~nucleotide~~



sequences of DNA fragments inserted into the plasmid pUC19, and synthesized a synthetic primer OBM #8 designed based on the nucleotide sequence of OBM cDNA were used. The sequences of these primers are shown as the Sequence SEQ ID No. NOs: 3 to No. 6 in the sequence table 6.

Furthermore, the determined nucleotide sequence of OBM cDNA is shown as Sequence SEQ ID No. NO: 22, and the deduced amino acid sequence determined therefrom is shown as the Sequence SEQ ID No. NO: 1.

<[Example 9]>

#### **Expression of the Protein Encoded by the cDNA of the Present Invention**

Plasmid pOBM291 was transfected into COS-7 cells with lipofectamine in each well of a 6-well plate using Lipofectamine, and the transfected COS-7 cells were cultured for two days in DMEM medium containing 10% fetal bovine serum for 2 days. The medium was replaced with a cysteine-/methionine-free DMEM (Dainippon-Seiyaku Co. DAINIPPON PHARMACEUTICAL CO., Ltd. LTD.) (800 µl/well) containing in which 5% dialyzed fetal bovine serum. The (800 µl/well) was added, and the cells were cultured for another 15 minutes. Then, followed by the addition of 14 µl of Express Protein Labeling Mix (NEN CO., LTD., 10 mCi/ml, manufactured by NEN Co.) was added thereto. After culturing the cells were cultured for four hours, 200 µl of DMEM including medium containing 10% fetal bovine serum was added, and the cells were cultured for 1 hour. After one hour culturing, the cells were washed twice with PBS. Then, 0.5 ml of a-TSA buffer (10 mM Tris-HCl (pH 8.0) containing 0.14 M NaCl, and 0.025% NaN<sub>3</sub>), containing 1% Triton X-100, 1% bovine hemoglobin, 10 µg/ml leupeptin, 0.2 TIU/ml aprotinin, and 1 mM PMSF, was added, and the mixture was allowed cells were left to stand for one hour on ice for 1 hour. Cells After the cells were disrupted crushed by pipetting, centrifugation was carried out at 3,000 xg at 4°C and centrifuged at 3000 x g for 10 minutes at so 4°C as to obtain a supernatant. To 100 µl of this supernatant, 200 µl of dilution buffer (TSA buffer containing 0.1% Triton X-100, 0.1% bovine hemoglobin, 10 µg/ml leupeptin, 0.2 TIU/ml aprotinin, and 1 mM PMSF) was added to 100 µl of this, and the resulting supernatant. The mixture was shaken for one hour at 4°C together with pProtein A Sepharose® (50 µl). The resultant

~~mixture was~~ at 4°C for 1 hour, and then centrifuged at ~~1500 x~~ 4°C and 1,500 X g for ~~one~~ 1 minute at ~~so~~ 4°C as to collect a supernatant. ~~Thereby, and thereby~~ a fraction(s) which is non-specifically adsorbed nonspecifically binding to the Protein A Sepharose® was removed. OCIF (1 µg) was added to this supernatant, and the ~~mixture~~ obtained supernatant was shaken at 4°C for ~~one~~ 1 hour ~~to achieve the binding of so that OBM bound OCIF to OBM.~~ ~~Anti~~ Then, an anti-OCIF polyclonal antibody (50 µg) was added, and the ~~mixture~~ solution was shaken at 4°C for ~~one~~ 1 hour ~~at 4°C.~~ Then, Protein A Sepharose® (10 µl) was further added, and the ~~mixture~~ solution was further shaken at 4°C for ~~an another additional~~ 1 hour ~~at 4°C, followed by centrifuge.~~ The solution was centrifuged at 1,500 xg at ~~1500 x~~ 4°C for 1 minute at 4°C ~~to collect and the precipitated fraction was collected.~~ The precipitate. ~~The precipitate~~ resulting from centrifugation at 1,500 xg at 4°C for 1 was washed twice with the dilution buffer, twice with ~~a the~~ dilution buffer without bovine hemoglobin-free dilution buffer, once with TSA buffer, and once with 50 mM Tris-HCl (pH 6.5). After washing, SDS buffer (0.125 M Tris-HCl, 4% dodecyl sodium dodecyl sulfate, 20% glycerol, 0.002% ~~B~~ bromophenol ~~B~~ blue, pH 6.8) containing 10% β-β mercaptoethanol was added to the precipitate. The ~~mixture~~ precipitate was heated at 100°C for 5 minutes ~~at 100°C~~ and subjected to SDS-PAGE (12.5% polyacrylamide gel, Daiichi Chemical Pure Chemicals Co., Ltd.). ~~The~~ After the gel was fixed ~~according to in accordance with a conventional commonly used method.~~ ~~Isotope~~, signals of isotope were amplified using with Amplify™ (Amersham Co., Ltd.), and the sample fixed gel was exposed to ~~Bio-Max~~ BioMax® MR film (~~KODAK~~ Kodak Co., Ltd.) at -80°C. The results are shown in Figure 8, ~~which indicates that~~ Fig. 8. The molecular weight of the protein encoded by the cDNA of the present invention has a molecular weight of was found to be about 40,000.

<[Example 10]>

#### **Binding of the pProtein. eEncoded by the cDNA of the pPresent iInvention to OCIF**

Plasmid pOBM291 was COS-7 cells were transfected into COS cells with plasmid pOBM291 with lipofectamine in each well of a 24-well plate using Lipofectamine. After culturing and cultured for 2- to 3 days. Then, the cells were washed with serum-free DMEM ~~culture medium.~~, and 200 µl of ~~culture medium for the binding assay~~ experiment (serum-free DMEM ~~culture medium~~ containing 0.2% calf bovine serum;

albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin, and 0.2% NaN<sub>3</sub>), supplemented with containing 20 ng/ml of <sup>125</sup>I-labeled OCIF, was added to the wells thereto. To the other wells Furthermore, 200 µl of culture the medium for the binding assay to which experiment containing 8 µg/ml of unlabelled OCIF had been added, in addition to 20 ng/ml of the <sup>125</sup>I-labeled OCIF, was added was added to other wells. After culturing for one hour at 37°C The cells were cultured in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>), the cells were at 37°C for 1 hour, and washed twice with 500 µl of phosphate buffered saline solution containing 0.1 mg/ml of heparin. Then. After washing, 500 µl of 0.1 N NaOH solution was added to each well, and then the plate well was allowed left to stand for 10 minutes at room temperature for 10 minutes so as to dissolve lyse the cells. The amount of <sup>125</sup>I in each well was measured by with a gamma counter. As a result, as shown in Figure 9, it was confirmed that the <sup>125</sup>I-labeled OCIF was found to bind bound only to the cells in which transfected with the plasmid pOBM291 was transfected 291 as shown in Fig. 9. In addition Further, it was also confirmed that the binding was confirmed to be conspicuously significantly inhibited by the addition of (unlabeled) OCIF at a 400-fold higher concentration of (unlabeled) OCIF. These From these results have demonstrated, it was revealed that the OBM, a protein OBM encoded by the cDNA of the plasmid pOBM291/291 specifically binds to bound OCIF on the surface of the transfected COS-7 cells.

<[Example 11]>

#### **Crosslinking Experiment of <sup>125</sup>I-labeled OCIF and to the pProtein. eEncoded by the cDNA of the pPresent. iInvention**

Crosslinking of <sup>125</sup>I-labeled monomer type OCIF and the protein encoded by the cDNA of the present invention was carried out In order to investigate analyze the characteristics of the protein encoded by the cDNA of the present invention in further detail more specifically, <sup>125</sup>I-labeled monomer type OCIF was allowed to crosslink with the protein encoded by the cDNA of the present invention. After transfection of COS-7 cells were transfected with plasmid pOBM291 into COS-7 cells according to in accordance with the method used described in the Example 8 -(3), 200 µl of the culture medium for the binding assay, as described above, experiment containing the above <sup>125</sup>I-labeled OCIF (25 ng/ml) was added to the wells thereto. The Furthermore, culture the

medium for the binding assay to which unlabeled OCIF at experiment containing a 400-fold higher concentration ~~was added~~ of unlabeled OCIF in addition to the <sup>125</sup>I-labeled OCIF was added to the other wells. ~~After culturing for one hour at 37°C~~ The cells were cultured in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>), ~~the cells were at 37°C for 1 hour and~~ washed twice with 500 µl of phosphate buffered saline solution containing 0.1 mg/ml of heparin. ~~Five hundred~~ 500 µl of phosphate buffered saline solution containing 100 µg/ml of a crosslinking agent, DSS (dissuccinimidyl suberate, ~~manufactured by~~ Pierce Co., Ltd.) was added to these cells, ~~followed by a reaction and the cells were allowed to react at 0°C for 10 minutes at 0°C.~~ The After the reaction, the cells in these wells were washed twice with 1 ml of cold-phosphate buffered saline (solution cooled to 0°C). ~~After the addition of~~ Then, 100 µl of 20 mM Hepes buffer containing 1% Triton X-100 (Wako Pure Chemicals Co. ~~Chemical Industries, Ltd.~~), 2 mM PMSF (Pphenylmethylsulfonyl fluoride, Sigma Co.), 10 µM Pepstatin (~~Wako Pure Chemicals Co., Ltd.~~), 10 µM Leupeptin ~~pepstatin~~ (Wako Pure Chemicals Co. ~~Chemical Industries, Ltd.~~), 10 µM leupeptin (Wako Pure Chemical Industries, Ltd.), 10 µM antipain (Wako Pure Chemicals Co. ~~Chemical Industries, Ltd.~~) and 2 mM EDTA (Wako Pure Chemicals Co. ~~Industries, Ltd.~~) was added to these cells, and the wells were allowed left to stand for 30 minutes at room temperature for 30 minute so as to dissolve the cells. ~~Fifteen~~ After 15 µl aliquots of these samples were heated in the presence of treated with SDS under non-reducing conditions according to in accordance with a conventional commonly used method and, they were subjected to electrophoresis with gel for SDS-electrophoresis using (gradient of 4- to 20% polyacrylamide-gradient gel (~~Daiichi Pure Chemical Co., DAIICHI PURE CHEMICALS CO., Ltd~~ LTD.)). After the electrophoresis, the gel was dried and exposed for 24 hours at -80°C to a BioMax® MS film (Kodak Co., Ltd.) using a with BioMax® MS sensitization amplifying screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films ~~was~~ were developed according to in accordance with a conventional commonly used method. As a result, a band with a molecular weight of a range of 90,000-110,000, shown in Figure 10, was detected by results of crosslinking of the <sup>125</sup>I-labeled monomer type OCIF and with the protein encoded by the cDNA of the present invention, a band having a molecular weight of about 90,000 to 110,000 was detected as shown in Fig. 10.

<[Example 12]>

### **Northern blotting-Blot aAnalysis**

ST2 cells were cultured to become confluent in a 25 -cm<sup>2</sup> T flask, for ~~attached-~~  
~~cell cultures were~~ culturing adherent cells, and treated with trypsin and. After being  
stripped from the T flask. ~~After washing,~~ the cells were washed and seeded ~~into~~ a 225 -  
cm<sup>2</sup> T flask ~~and cultured for 4 days in a CO<sub>2</sub> incubator with .~~ 60 ml of an  $\alpha$ -MEM  
culture-medium containing 10<sup>-8</sup> M of the active-form of vitamin D<sub>3</sub>, 10<sup>-7</sup> M  
dexamethasone, and 10% bovine fetal bovine-serum was added thereto, and the cells  
were cultured in a CO<sub>2</sub> incubator for 4 days. ~~Total~~ Then, total RNA was extracted from  
the above-cultured ST2 cells using with ISOGEN (Wako Pure Chemicals Co., Industries,  
Ltd.). ~~The~~ In addition, total RNA was ~~also extracted in the same manner from ST2 cells~~  
~~which were~~ cultured in the absence of the active-form of vitamin D<sub>3</sub> and dexamethasone.  
~~After in accordance with the addition of above method.~~ To 20  $\mu$ g (4.5  $\mu$ l) of each total  
RNA sample, 2.0  $\mu$ l of 5X gel electrophoresis buffer ~~solution~~ (0.2 M morpholine  
~~propane sulfoni~~ morpholinopropanesulfonic acid; (pH 7.0; 7.0), 50 mM sodium acetate, 5  
mM EDTA) ~~and,~~ 3.5  $\mu$ l of formaldehyde; and 10.0  $\mu$ l of formamide ~~to 20  $\mu$ g (4.5  $\mu$ l)~~  
~~of each of the total RNAs, the mixtures were added.~~ The total RNA samples were  
incubated at 55°C for 15 minutes ~~at 55°C~~ and subjected to electrophoresis. ~~The gel~~ Gels  
for electrophoresis ~~was prepared according to the formulation consisted~~ of 1.0% agarose,  
2.2 M ~~deionized~~ ionized formaldehyde, 40 mM ~~morpholinopropane~~  
~~sulfoni~~ morpholinopropanesulfonic acid (pH 7.0), 10 mM sodium acetate; and 1 mM  
EDTA. ~~The~~ Moreover, the electrophoresis was ~~carried out~~ performed in a buffer solution  
~~of comprising~~ 40 mM ~~morpholino propane sulfoni~~ morpholinopropanesulfonic acid; (pH  
7.0; 7.0), 10 mM sodium acetate; and 1 mM EDTA. After the electrophoresis, the RNA  
was transferred ~~entoto~~ to nylon membranes. About 1.0 kb DNFA fragments ~~was were~~  
obtained by ~~digesting~~ cleaving pOBM291 with a restriction enzyme, EcoRI.  
Hybridization ~~was carried out using this DNA fragment, and~~ labeled with a Megaprime  
DNA-labeling kit (Amersham Co.) and  $\alpha$ -<sup>32</sup>P-dCTP (Amersham Co.), Ltd.) using  
MEGAPRIME DNA Labeling Kit (Amersham Co., Ltd.), and thus used as a ~~probe~~ probes  
for hybridization. As a result, as shown in Figure 11, it was ~~confirmed~~ revealed that when  
ST2 cells were cultured in the presence of active form vitamin D<sub>3</sub> and dexamethasone,

gene expression of the protein (OBM) encoded by the cDNA of the present invention (OBM) was isstrongly induced strongly in the ST2 cells cultured in the presence of the active-form of vitamin D<sub>3</sub> and dexamethasone.

<[Example 13]>

# Osteoclasts formation supporting capabilityAbility of the pProtein eEncoded by the cDNA of the present inventionPresent Invention to Support Osteoclast Formation

~~pOBM291~~ was transfected into COS cells according to ~~In accordance with the same method described in the Example 8(3)-(3),~~ COS-7 cells were transfected with ~~pOBM219~~. After ~~three3-day daysincubation, trypsinizedthe~~ cells were ~~treated with trypsin and then centrifuged-washed~~ once with phosphate buffered saline solution ~~by centrifugation~~. Then, ~~then~~the cells were fixed with ~~at room temperature for 5 minutes in suspension of PBS containing 1% paraformaldehyde at room temperature for 5 minutes,~~ followed by ~~washing with PBSand then centrifuged-washed~~ six times ~~bywith centrifugationPBS~~. 700  $\mu$ l of  $1 \times 10^6$  /ml mouse Mouse spleen cells and 350  $\mu$ l of  $4 \times 10^4$  /ml ST2 cells ~~which were suspended in a~~ prepared with  $\alpha$ -MEM culture medium containing  $10^{-8}$  M of the active-form of vitamin D<sub>3</sub>,  $10^{-7}$  M dexamethasone, and 10% bovine fetal ~~bovine-serum,~~ were so that the cell concentration become  $1 \times 10^6$  cells/ml or  $4 \times 10^4$  cells/ml and then added to a 24-well plate ~~in a volume of 700  $\mu$ l and 350  $\mu$ l, respectively.~~ Furthermore, TC insert (Nunc Co., Ltd.) was set in each well. The ~~above-described-fixed COS cells (350  $\mu$ l) diluted to various concentrationsstepwise with the above-mentioned culture medium and OCIF solution-(50  $\mu$ l),~~ were added to the TC inserts and cultured at 37°C for 6 days ~~at 37°C~~. As a result, it was ~~confirmedrevealed~~ that ~~the osteoclasts formation-inhibitivean~~ activity of OCIF ~~can be inhibitedto inhibit~~ osteoclast formation was suppressed by the protein encoded by the cDNA of the present invention.

<[Example 14]>

## Expression of sSecretedory-form-type OBM

### (1) Construction of a-plasmidPlasmid for the expression of secretedExpressing Secretory-formType OBM Expression

A PCR reaction was carried out using OBM HF (~~Sequence Table, SequenceSEQ ID No. NO: 7)~~ and /OBM XR (~~Sequence Table, SequenceSEQ ID No. NO: 8)~~ and

pOBM291 as primers and pOBM291 as a template, respectively. After purification by the reaction product was purified through agarose gel electrophoresis, the product was it was digested with the restriction enzymes, HindIII and EcoRI, and further then purified by through agarose gel electrophoresis again. The purified fragment (0.6 kb), Hind III/HindIII/EcoRI fragment (5.2 kb) of pSec TagA (Invitrogen Co., Ltd.) and EcoRI/PmaeCI fragment (0.32 kb) of OBM cDNA were ligated using a was subjected to ligation kit verusing Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.), and subsequently *E. Escherichia coli* DH5- $\alpha$  was transformed using by the reaction ligation product. Plasmids werewas purified by means of alkali-SDS method from the resulting obtained ampicillin - resistant strains and digested by alkaline-SDS method and then cleaved with restriction enzymes so as to select a plasmid with fragments of a length of wherein 0.6 Kbbk and 0.32 kb being of fragments were inserted into pSec TagA. Selected The selected plasmid was identified as having asubjected to sequencing with Dye Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.), thereby it was confirmed that the plasmid had the sequence encoding the secreted secretory-form type OBM (nucleotide sequence: nucleotides 338-1355 in of Sequence SEQ ID No. NO: 2, amino acid sequence: acids 72-316 in the Sequence of SEQ ID No. NO: 1) by sequencing using a dyeterminator cycle sequencing FS kit (Perkin Elmer Co.). This After the plasmid was digested with restriction enzymes, NheI and XhoI to isolate, a fragment (1.0 kb) containing the corresponding to secretedory-form type OBM cDNA was collected by agarose gel electrophoresis. This fragment was inserted into thea NheI/XhoI fragment (10.4 kb) of and digested expression vector, pCEP4 (10.4 kb) (Invitrogen Co., Ltd.), using a, by using the ligation kit, and *Escherichia E. coli* DH5- $\alpha$  was $\alpha$  were transformed using with the reaction ligation product thereof. Plasmids werewas purified by alkali-SDS method, from the resulting ampicillin -resistant strains obtained, by alkaline-SDS method, and digested with the restriction enzymes. Then the plasmid was analyzed so as to select an *Escherichia E. coli* strain having the secreted form strains which had a plasmid for expressing secretory-type OBM expression plasmid-(pCEP sOBM) with the correct desired structure. The An *Escherichia E. coli* strain containing having the pCEP sOBM was cultured, and the pCEP sOBM was purified using therefrom with QIA-filter plasmid midi kit @ Filter Plasmid Midi Kit (QIAGEN Co CO., LTD.).

(2) Expression of sSecretedory-formType OBM

293-EBNA cells were suspended in IMDM containing 10% FCS (IMDM-10% FCS), and seeded ~~into~~ in a collagen-coated 24- well plate-coated with collagen (manufactured by Sumitomo Bakelite Co SUMITOMO BAKELITE CO., LtdLTD.) ~~in aso~~ that the cell density concentration of was  $2 \times 10^5$  cells/2 ml/well, and cultured overnight. The cells were transfected with 1  $\mu$ g of pCEP sOBM or pCEP4 using 4  $\mu$ l of Lipofectamine (Gibco Co.). After culturing for two days (GIBCO CO., LTD.), and then cultured in 0.5 ml of a-serum-free IMDM or IMDM-10% FCS FCS for another 2 days. Thereafter, the conditioned medium was collected. Expression of ~~the secreted~~ secretory- ~~formtype~~ OBM in the conditioned medium was confirmed as follows in the following manner. Sodium hydrogen carbonate After sodium hydrogencarbonate was added to the conditioned medium ~~to aso~~ that the final concentration ~~of was~~ 0.1 M ~~and,~~ the culture solution was added to a 96-well plate. ~~The plate was allowed, and left~~ to stand at 4°C overnight ~~at 4°C, thereby immobilizing.~~ Then the OBM in the conditioned medium ~~on was immobilized in~~ the 96-well plate. ~~The plate was filled with a Block Ace™~~ This plate was left to stand for blocking at room temperature for 2 hours by use of BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted ~~four fold~~ with PBS to be one forth concentration (B-PBS) ~~and allowed to stand for two hours at room temperature to block residual binding sites of the plate.~~ After the addition to each well of 100 ~~Then, 100~~  $\mu$ l of 3-100 ng/ml of OCIF which was diluted with B-PBS, the plate was allowed was added to each well, and the wells were left to stand for two hours at 37°C; followed by for 2 hours. After washing the plate with PBS containing 0.05% Tween 20 (PBS-T). ~~Then, 100, 100~~  $\mu$ l of a peroxidase-labeled rabbit-anti-OCIF rabbit polyclonal antibody, which was described in WO 96/26217 ~~which was 26217,~~ diluted with B-PBS was added to each well. ~~After allowing, and the cells were left~~ to stand at 37°C for two 2 hours ~~at 37°C, the wells were washed six times.~~ After washing each well with PBS-T: ~~Then six times,~~ a 100  $\mu$ l of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added ~~in the amount of 100  $\mu$ l per well thereto~~ and allowed then left to stand at room temperature for about 10 minutes. Thereafter, ~~whereupon the reaction was terminated by the addition of 100  $\mu$ l of a termination solution (Stopping Reagent;~~ (Scytek Co., Ltd.) was added to each well. Absorbance The absorbance of each well at



450 nm of each well was measured by with a microplate reader. The results are shown in Figure 12. In the plate in which substances included in the conditioned medium of the cells transduced by pCEP sOBM were immobilized, absorption at 450 nm increased according to the OCIF concentration of the added OCIF in dependent manner. On the other hand, in the plate in which substances included in the conditioned medium of the cells transfected with pCEP sOBM was immobilized. On the other hand, transduced by pCEP4 vector were immobilized, no increase in absorbance at 450 nm was seen in the plate in which the conditioned medium of the cells transfected with vector pCEP4 was immobilized. Furthermore, Fig. 13 shows the results of an experiment wherein experiments when the proportion amount of the conditioned medium which is used for applied to the immobilization was changed varied within a range of 5- to 90% and a specific constant concentration of OCIF (50 ng/ml) was further added. It can be seen that in the absorbance plate in which substances included in the conditioned medium of the cells transduced by pCEP sOBM were immobilized, absorption at 450 nm increased according corresponding to the an increase in the amount of the proportion of conditioned medium. On the conditioned medium in other hand, in the plate wherein plate in which substances included in the conditioned medium of the cells transfected with pCEP sOBM was immobilized, whereas transduced by pCEP4 vector were immobilized, no such increase in absorbance was seen in the plate wherein the conditioned medium of the cells transfected with vector pCEP4 was immobilized absorption was observed. From these results, it secretory-type OBM was confirmed that secreted form OBM is to be produced in the conditioned medium of the cells transfected with by pCEP sOBM.

<[Example 15]>

#### Expression of Thioredoxin-OBM Fusion Protein (Trx-OBM)

##### (1) Construction of a thioredoxin Vector for Expressing Thioredoxin-OBM Fusion Protein (Trx-OBM)-expression vector

10 µl of 10X ExTaq buffer (Takara Shuzo Co. TAKARA SHUZO CO., LTD.), 8 µl of 10 mM dNTP (Takara Shuzo Co. dNTPS (TAKARA SHUZO CO., LTD.), 77.5 µl of sterilized distilled water, 2 µl of an aqueous pOBM291 solution of

pOBM291 (10 ng/ $\mu$ l), 1  $\mu$ l of primer OBM3 (100 pmol/ $\mu$ l, Sequence Table, Sequence SEQ ID No. NO: 9), 1  $\mu$ l of primer OBMSalR2 (100 pmol/ $\mu$ l, Sequence Table, Sequence SEQ ID No. NO: 10); and 0.5  $\mu$ l of ExTaq (5-u/ $\mu$ l) (Takara Shuzo Co., Ltd.) were mixed together, and reacted then (PCR reaction) was conducted in a microtube-centrifugal tube for centrifugation. After reacting the reaction was carried out at 95°C for 5 minutes, at 50°C for one second, at 55°C for one minute, at 74°C for one second; and at 72°C for 5 minutes, at the cycle reaction consisting of a reaction at 96°C for one minute, at 50°C for one second, at 55°C for one minute, at 74°C for one second; and at 72°C for 3 minutes; was repeated 25 times. From the total reaction liquid-After gel electrophoresis through 1% agarose, an approximately 750 bp DNA fragment of about 750 bp was purified by 1% agarose gel electrophoresis using from the whole reaction solution with QIAEX® II gGel eExtraction kKit (QIAGEN Co., Ltd.). The whole amount All of the purified DNA fragment was digested/cleaved with restriction enzymes SalI and EcoRI (Takara Shuzo Co., Ltd.), and subjected to an 1.5% agarose gel electrophoresis to purify a DNA fragment of about 160 bp (Fragment 1), which was (fragment 1) was purified by gel electrophoresis through 1.5% agarose and dissolved in 20  $\mu$ l of sterilized distilled water. In the same manner Similarly, a DNA fragment of about 580 bp (Fragment 2) obtained by digesting 4  $\mu$ g of pOBM291 and 2  $\mu$ g of pTrxFus (Invitrogen Co., Ltd.) were cleaved with restriction enzymes BamHI and BamHI/EcoRI and BamHI/SalI (Takara Shuzo Co., Ltd.) and a, respectively. A DNA fragment of about 580-bp (fragment 2) and an approximately 3.6 -kb DNA fragment (Ffragment 3) obtained by digesting 2  $\mu$ g of pTrXFus (Invitrogen Co.) with restriction enzymes BamHI and SalI (Takara Shuzo Co.) were respectively purified therefrom, respectively, and dissolved in 20  $\mu$ l of sterilized distilled water. The QIAEXII gel extraction kit QIAEX® II Gel Extraction Kit was used for for purifying the purification of DNA fragments. Fragments 1, 2 and 3 were ligated by incubating them using DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.) at 16°C for 2.5 hours using DNA ligation kit ver. 2 (Takara Shuzo Co.). Using the ligation reaction liquid Then, Escherichia *E. coli* strain-GI724 cells (Invitrogen Co., Ltd.) was/were transformed according to with the ligation product in accordance with the method described in the Instruction Manual of an instruction manual attached to ThioFusion Expression System (Invitrogen Co., Ltd.). A

~~microorganism strain with~~ Among the resulting ampicillin-resistant transformants, one having a plasmid, in which the ~~an~~ OBM cDNA fragment (nucleotide sequence: 350-1111 in the Sequence of SEQ ID No. NO: 2, amino acid sequence corresponding to: 76-316 in the Sequence of SEQ ID No. NO: 1) ~~is fused in frame~~ was linked to a thioredoxin gene in the same reading frame and was selected from the resulting ampicillin-resistant transformants by the ~~after~~ analysis of restriction maps. DNA fragment map obtained by digestion with restriction enzymes ~~enzyme cleavage~~ and DNA sequence determination ~~sequencing~~. The ~~microorganism obtained~~ strain thus obtained was named ~~referenced~~ as GI724/pTrxOBM25.

(2) Expression of OBM in *Escherichia coli*

The GI724/pTrxOBM25 strain and the GI724 ~~containing~~ strain having pTrxFus (GI724/pTrxFus) were respectively cultured six hours with shaking at 30°C in 2 ml of RMG-Amp culture medium (0.6% Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.05% NaCl, 0.1% NH<sub>4</sub>Cl, 1.2% casamino acid (Difco Co., Ltd.), 1% glycerol, 1 mM MgCl<sub>2</sub>, and 100 µg/ml ampicillin (Sigma Co., Ltd.), pH 7.4) with shaking at 30°C for 6 hours. The ~~broth~~ 0.5 ml of the ~~broth~~ cell suspension was added to 50 ml of Induction culture medium (0.6% Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.05% NaCl, 0.1% NH<sub>4</sub>Cl, 0.2% casamino acid, 0.5% glucose, 1 mM MgCl<sub>2</sub>, 100 µg/ml ampicillin, pH 7.4) and cultured with shaking at 30°C. When OD<sub>600nm</sub> reached about 0.5, L-tryptophan was added to ~~so that the~~ final concentration of ~~was~~ 0.1 mg/ml, followed by shaking when the culture value at OD<sub>600</sub> became about 0.5, and the cells were further cultured at 30°C for an additional 6 hours. The culture cell ~~broth~~ suspension was centrifuged at 3000 ~~3,000~~ × g to collect and the collected cells, which were then suspended in 12.5 ml of PBS (10 mM ~~phosphate~~ phosphoric acid buffer, 0.15 M NaCl, pH 7.4). The suspension was subjected to an ultrasonic generator ~~ultrasonication~~ using an ultrasonicator (Ultrasonics Co., Ltd.) to ~~so disrupt~~ that the cells. The disrupted cells were crushed and then centrifuged at 7000 ~~7,000~~ × g for 30 minutes to obtain a. The recovered supernatant liquid was used as a soluble protein fraction. Ten 10 µl of this soluble protein fraction solution was subjected to SDS polyacrylamide (10%) electrophoresis under reducing conditions. As a result, a band with having a molecular weight of about 40 kDa which was observed in the soluble protein fraction solution of GI724/pTrxOBM25, while not detected ~~observed~~ in the

soluble protein fraction solution of GI724/pTrxFus was found in the soluble protein fraction of GI724/pTrxOBM25 (Figure Fig. 14). Accordingly ~~Thus~~, it was confirmed that ~~the~~ thioredoxin-OBM fusion protein (Trx-OBM) ~~of thioredoxin and OBM~~ was expressed in ~~Escherichia~~ *E. coli*.

(3) Binding capability ~~Ability~~ of Trx-OBM to OCIF

~~Binding of~~ In the following experiment, it was confirmed that the expressed Trx-OBM bound to OCIF ~~was confirmed according to the following experiment~~. Anti-thioredoxin antibody (Invitrogen Co., Ltd.) ~~which was diluted to 5000-fold with 10 mM sodium hydrogen carbonate~~ hydrogencarbonate solution so that the concentration was 1/5,000. 100  $\mu$ l thereof was added to each well of a 96-well immunoplate (Nunc Co., Ltd.) in the amount of 100  $\mu$ l per well. ~~After being allowed and then left to stand at 4°C overnight at 4°C, the liquid in the wells~~. After the solution in each cell was discarded. ~~Two hundred, 200  $\mu$ l of a solution prepared by diluting Block Ace™ of 1/2 concentration of BLOCKACE (Snow Brand Milk Products Co., Ltd.) two-fold~~ diluted with PBS (BA-PBS) was added to each well. ~~After being allowed and then left to stand for one hour at room temperature, for 1 hour~~. After the solution was discarded and, 100  $\mu$ l of the soluble protein fractions originating from the above-described GI724/pTrxOBM25 or which was diluted stepwise with BA-PBS and 100  $\mu$ l of that derived from GI724/pTrxFus, each which was diluted stepwise with BA-PBS in various concentrations BPB were added to each well in the amount of 100  $\mu$ l. After being allowed wells and left to stand for two hours at room temperature, for 2 hours, respectively. After washing each well was washed well three times with PBS-T and charged with, 100  $\mu$ l of OCIF (100 ng/ml) which was diluted with BA-PBS. ~~After being allowed was added to each well and left to stand for two hours at room temperature, each for 2 hours~~. After washing each well was washed three times with PBS-T and charged with, 100  $\mu$ l of peroxidase- labeled rabbit anti- OCIF rabbit polyclonal antibody (described described in WO 96/26217)26217, which was diluted 2,000-fold with BA-PBS. ~~After being allowed so that the concentration was 1/2,000, was added to each well and left to stand for two hours at room temperature, each for 2 hours~~. After washing each well was washed six times with PBS-T and charged with, 100  $\mu$ l of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytex Co.). ~~After being~~

allowed, Ltd.) was added thereto and then left to stand at room temperature for about 10 minutes at room temperature. Thereafter, each well was charged with 100 µl of termination solution (Stopping Reagent; (Scytek Co., Ltd.) was added thereto. Absorbance of each well at 450 nm was measured by with a microplate reader. The results are shown in Figure Fig. 15. There was When the concentration of the soluble protein fraction solution derived from GI724/pTrxFus increased, the absorbance increased in a concentration (of the added solution)-dependent manner, while no difference in absorbance was observed between the sample with when the soluble protein fraction originating solution derived from GI724/pTrxFus was added thereto and the sample without the addition of this when said soluble protein fraction solution was not added. On the other hand Furthermore, the absorbance increased in the samples to which the soluble protein fraction originating from GI724/pTrxOBM25 was added in proportion to the concentration of the soluble protein fraction Fig. The 16 shows the results of the other experiment wherein experiments when the dilution rate of the soluble protein fraction solution was maintained kept constant (1%) while adding and OCIF diluted stepwise with BA-PBS in different concentrations (0-100 ng/ml) are shown in Figure 16. was further added. It can be seen that the absorbance remained Absorbance was kept low at any concentrations regardless of the concentration of OCIF in samples using a when soluble protein fraction originating solution derived from GI724/pTrxFus was added. However, whereas the absorbance was increased in proportion to the an OCIF concentration in the samples to which the dependent manner when soluble protein fraction originating solution derived from GI724/pTrxOBM25 was added. Based on these results Thus, it was confirmed that the Trx-OBM which is produced from in GI724/pTrxOBM25 has a capability of binding had an ability to bind OCIF.

#### (4) Large-scale cultivation Culture of Escherichia E. coli which produces Producing Trx-OBM

GI724/pTrxOBM25 cells were spread on an RMG-Amp agar medium (0.6% Na<sub>2</sub>-POHPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.05% NaCl, 0.1% NH<sub>4</sub>Cl, 2% casamino acid, 1% glycerol, 1 mM MgCl<sub>2</sub>, 100 µg/ml ampicillin, 1.5% agar, pH 7.4) using with a platinum transfer loop. The cells were and cultured at 30°C overnight at 30°C. The cultured cells were suspended in 10 ml of Induction medium. The 5 ml of the suspension was added 5 ml for

each to two of 2-l Erlenmeyer L conical flasks containing 500 ml of Induction medium and cultured at 30°C with by shaking at 30°C. When the OD<sub>600nm</sub> reached about 0.5, L-tryptophan was added to so that the final concentration of was 0.1 mg/ml. Culturing with when OD<sub>600</sub> value became about 0.5, and then the cells were further cultured by shaking was at continued 30°C for six 6 hours at 30°C. The culture cell broth suspension was centrifuged at 3,000 X g for 20 minutes at 3000 x g to collect, and the cells, which were collected and then suspended in 160 ml of PBS. The suspension was subjected to ustrasoniaction using an ultrasonic generator ultrasonicator (Ultrasonics Co., Ltd.) to disrupt the for crushing cells. The supernatant liquid was, and then centrifuged at 7,000 X g for 30 minutes at 7000 x g to obtain a. Thereafter, the supernatant was recovered as soluble protein fraction.

(5) Preparation of OCIF-immobilized aAffinity eColumn

Two 2 g of TSKgel AF-Tolresyl ToyopaTOYOPAL 650 (Tosoh Corp Toso Co., Ltd.) and 40 ml of 1.0 M potassium phosphate buffer (pH 7.5) containing 35.0 mg of recombinant OCIF, which was prepared aeedding to the by a method described in WO 96/26217, 26217 were mixed. The mixture was together and gently shaken at 4°C overnight at 4°C to effect so as to cause a coupling reaction. The reaction mixture was centrifuged to remove After the supernatant. To inactivate exeess active residues was removed by centrifugation, 40 ml of 0.1 M Tris-HCl hydrochloric acid buffer (pH 7.5) was added to the precipitated carrier, and the mixture was gently shaken at room temperature for one 1 hour. The carrier, in a column was washed order to inactivate an excess amount of active groups thereon. After washing the column with both 0.1 M glycine-HCl hydrochloric acid buffer (pH 3.3) containing 0.01% Polysorbate 80 and /0.2 M NaCl and 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80 and /0.2 M NaCl. The carrier in the column was equilibrated by charging, the column was washed twice with 10 mM sodium phosphate buffer (pH 7.4) containing 0.01% Polysorbate 80 80 and equilibrated therewith.

(6) Purification of Trx-OBM aUsing OCIF-i mmobilized aAffinity eColumn

Unless otherwise indicated, purification Purification of Trx-OBM was carried out at 4°C unless otherwise stated. The above-mentioned OCIF-immobilized affinity carrier (10 ml) and the above soluble protein fraction solution (120 ml) prepared described in

Example 15-(4) were mixed together. The mixture was gently shaken ~~overnight~~ at 4°C in four 50- ml centrifuge tubes using with a rotor at 4°C overnight. ~~An~~ The carrier in the mixture was embedded in Econo-column™ Column (Bio-Rad Co., Ltd., internal diameter: 1.5 cm, length: 15 cm, manufactured by BioRad Co.) was filled with the carrier in the mixture. The column was ~~charged~~ washed with 300 ml of PBS containing 0.01% Polysorbate 80, 100 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 0.01% Polysorbate 80 and 2 M NaCl, and 100 ml of 0.1 M glycine-HCl hydrochloric acid buffer (pH 3.3) containing 0.01% Polysorbate 80 and 0.2 M NaCl, ~~in that order.~~ Next, proteins adsorbed in Then, protein was eluted from the column ~~were eluted~~ with 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80 and 0.2 M NaCl. The 5 ml eluate was fractions were collected in 5 ml portions. ~~Each fraction thus collected was immediately neutralized with addition~~ Immediately, a 10% volume of 2 M Tris buffer solution (pH 8.0) was added for neutralization. ~~Presence~~ The presence or absence of Trx-OBM in each fraction of the eluted fractions eluate was determined according to examined in accordance with the above method previously as described in Example 15-(3) (the binding capability ability to bind OCIF). ~~The fractions~~ Fractions containing Trx-OBM were collected and further purified further.

(7) Purification of Trx-OBM by gGel fFiltration

~~About~~ Using Centriplus® 10 and Centricon® 10 (Amicon Co., Ltd.), about 25 ml of the above Trx-OBM fractions obtained in fraction of Example 15 -(6) was concentrated by centrifugation to a final volume of about 0.5 ml by centrifuge using Centriplus 10 and Centricon 10 (Amicon Co.). This sample was ~~applied~~ subjected to a Superose® 12 HR 10/30 column (1.0 × 30 cm, Pharmacia Co., Ltd.) previously equilibrated with PBS containing 0.01% Polysorbate 80. ~~For the separation,~~ The column was developed using PBS containing 0.01% Polysorbate 80 ~~was used~~ as a mobile phase at a flow rate of 0.25 ml/min. ~~The~~ and 0.25 ml eluate fractions were collected from the column ~~was collected in 0.25 ml portions.~~ The Trx-OBM in the thus collected fractions was detected by the same method as previously described in Example 15-(3) and by SDS-polyacrylamide electrophoresis (gradient gel of 10- to 15% polyacrylamide gel, Pharmacia Co., Ltd.) using Phast System (Pharmacia Co., Ltd.) and silver staining. Fractions (Fr. 20- to 23) containing purified Trx-OBM were collected and ~~the~~ subjected to measurement of Trx-

OBM protein concentration of Trx-OBM was determined. The measurement of the protein concentration was carried out with DC-protein assay kit (Bio-Rad Co., Ltd.) using bovine serum albumin as a standard substance using DC Protein assay kit (BioRad Co.).

<[Example 16]>

#### Osteoclast formation inducing activity of OBM

#### Osteoclastogenesis Promoting Activity of OBM

COS-7 cells were transfected with pOBM291 and pcDL-SR  $\alpha$ 296 were respectively transfected into COS-7 cells using Lipofectamine (Gibco BRL Co., Ltd.), respectively. The After the cells were cultured in DMEM containing 10% FCS for one day, trypsinized they were treated with trypsin, plated on and seeded in a 24-well plate, in which a cover slip glass (15 mm round-shape, manufactured by Matsunami Co., Ltd.) in 24-well plates at was seated, so that the concentration became  $5 \times 10^4$  cells-per-/well, and. The cells were then cultured for 2 another two days. The After washing the culture plate was washed once with PBS. The cells were fixed with, PBS containing 1% paraformaldehyde was added thereto, and the cells were incubated at room temperature for 8 minutes and fixed. The After washing the plate on in which the fixed cells were attached was washed 6 fixed six times with PBS, then 700  $\mu$ l of  $1 \times 10^6$  cells/ml suspension of mouse spleen cells suspended at  $1 \times 10^6$ /ml cell in  $\alpha$ -MEM containing  $10^{-8}$  M of the active-form of vitamin D<sub>3</sub>,  $10^{-7}$  M dexamethasone, and 10% bovine fetal bovine serum werewas added to each well cell. A Millicell® PCF (Millipore Co., Ltd.) was set in on each well, and a 700  $\mu$ l of  $4 \times 10^4$  cells/ml suspension of ST2 cells in the above-mentioned culture medium ( $4 \times 10^4$ /ml) werewas added, 700  $\mu$ l per well, into to the Millicell® PCF followed by incubation and cultured at 37°C for 6 days. After the culture, the Millicell® PCF was removed, and the plate was washed once with PBS, and the. The cells were fixed with acetone-ethanol solution (50:50) for one 1 minute. Then, and then only the cells exhibiting showing tartaric acid- resistant acid phosphatase activity (TRAP activity), which is a specific marker for osteoclasts, were selectively stained using LEUKOCYTE ACID PHOSPHATASE with leukocyte acid phosphatase kit (Sigma Co., Ltd.). As a result of microscopic observation, TRAP positive cells were not detected in the wells in which COS-7 cells transfected with pcDL-SR  $\alpha$ 296 were fixed. In contrast using a microscope,  $45 \pm 18$  (average  $\pm$  standard deviation, n = 3) TRAP positive cells



were observed in the wells in which ~~COS-7 cells~~ pOBM291-transfected COS-7 cells were fixed, while no cells showing TRAP activity were detected in the wells in which pcDL-SR  $\alpha$ 296-transfected with pOBM291 COS-7 cells were fixed.

~~Moreover~~ Furthermore, it was also confirmed that calcitonin bound to ~~these~~ said TRAP positive cells. ~~Based on these findings~~ Thereby, it ~~has been proven~~ was revealed that OBM ~~has~~ had an activity to promote osteoclast formation ~~indueing activity~~.

<[Example 17]>

#### Osteoclast formation-indueing activity **Osteoclastogenesis Promoting Activities of Trx-OBM and sSecretedory-formtype OBM**

Mouse spleen cells were suspended in  $\alpha$ -MEM containing  $10^{-8}$  M of the active-form of vitamin D<sub>3</sub>,  $10^{-7}$  M dexamethasone, and 10% bovine fetal ~~bovine~~-serum at in a concentration of  $2 \times 10^6$  cells/ml. ~~The~~, and 350  $\mu$ l of this suspension was added to each well of a 24 -well plate ~~in the amount of 350  $\mu$ l per well~~. Each well was then charged with 350  $\mu$ l of at the solution prepared (40 ng/ml) obtained by diluting the purified Trx-OBM with the above-mentioned culture medium (40 ng/ml), 350  $\mu$ l of solution prepared obtained by 10-fold diluting the conditioned medium ~~which was produced by culturing of 293-EBNA cells, in (which were transduced by pCEP sOBM or pCEP4 were transfected, cultured in IMDM-10% FCS, with the above-mentioned culture medium,)~~ so that the concentraion was 1/10, or 350  $\mu$ l only of the above-mentioned culture medium. ~~The~~ alone was added to each well. Then, a Millicell® PCF (Millipore Co., Ltd.) was set on each well, ~~to which~~ and 600  $\mu$ l of ST2 cells ~~which was suspended~~ 4 X 10<sup>4</sup> cells/ml suspension of ST2 cell in the above-mentioned culture medium ( $4 \times 10^4$ /ml) were added to the Millicell® PCF. After the cells were cultured for ~~six~~ 6 days, the Millicell® PCF was removed. ~~The~~, and the plate was washed once with PBS ~~and~~. When the cells were fixed with acetone-ethanol solution (50: 50) for ~~one~~ 1 minute. ~~Then~~, only the cells ~~exhibiting the~~ showing tartaric acid resistant acidie phosphatase activity (TRAP activity) were ~~selectively stained using with~~ LEUKOCYTE ACID PHOSPHATASE kit (Sigma Co., Ltd.). ~~The result of microscopic~~ Through observation revealed that no cells ~~exhibiting the TRAP activity were detected in the wells to which no Trx-OBM was added~~ under a microscope, whereas  $106 \pm \pm 21$  (average  $\pm \pm$  standard deviation, n = 3) TRAP- positive cells were observed in the wells ~~to which~~ when Trx-OBM was added,

while no cells showing TRAP activity were detected in the wells when not added.  
Similarly, ~~while no cells exhibiting TRAP activity were detected in the wells to which~~  
~~conditioned medium of 293-EBNA transfected with pCEP4 had been added, 120 ± 31~~  
(average ± standard deviation, n = 3) TRAP positive cells were observed in the wells  
~~to when which the~~ conditioned medium of 293-EBNA ~~transfected with pCEPsOBM had~~  
~~been cells transduced by pCEP-sOBM was added, while no cells showing TRAP activity~~  
were detected in the wells when not added. ~~Moreover~~ Furthermore, it was also confirmed  
that calcitonin binds to these TRAP positive cells. ~~These results have proven~~ Thereby,  
it was revealed that Trx-OBM and secreted ~~ory-form type~~ OBM ~~exhibit~~ had an activity to  
promote osteoclast formation-inducing activity.

<[Example 18]>

**Identity of the pProtein OBM eExpressed by the cDNA of the pPresent iInvention  
and ~~the natural type~~ Natural-Type OCIF- bBinding pProtein of the pPresent  
iInvention**

(1) —

(1) ~~p~~Preparation of rabbit anti-OBM of Anti-OBM Rabbit pPolyclonal aAntibody  
Three male Japanese white rabbits (weight: 2.5- to 3.0 kg, supplied purchased  
by from Kitayama Labesth Co., Ltd.) were immunized subjected to hypodermic  
immunization with the purified OBM (thioredoxin-OBM fusion protein) produced  
according to the method in Examples 14(6) and 14(7) by subcutaneously injecting 1  
ml/dose of emulsion prepared by mixing 200 µg/ml of the purified OBM (thioredoxin-  
OBM fusion protein), which was obtained in accordance with equal volume the methods  
described in Examples 14-(6) and 14-(7), with 200 µg/ml of Freund's complete adjuvant  
(~~DIFCO~~ Difco Co.), ~~six~~ Ltd.). The immunization was carried out 6 times, ~~once a~~ in total  
with one-week. ~~Ten days after~~ interval each, and all the blood was collected from the  
rabbits on the 10<sup>th</sup> day counted from the last immunization, ~~the rabbits were~~  
exsanguinated. Antibody An antibody was purified from the fractionated serum as  
follows in the following manner. ~~Ammonium sulfate was added to the~~ That is, the  
antiserum ~~which was~~ diluted two-fold with PBS to be 1/2 concentraion, and ammonium  
sulfate was added thereto so that the final concentration ~~of was~~ 40% (w/v%). ~~After being~~  
allowed Then, the antiserum was left to stand at 4°C for one 1 hour at 4°C, ~~the mixture~~

~~was and~~ centrifuged at 8,000 X g for 20 minutes ~~at 8000 x g to obtain a precipitate.~~  
~~The~~ Thereafter, the precipitate was collected and dissolved in a small ~~am~~ aliquot of PBS,  
and then dialyzed against PBS at 4°C, ~~and loaded to.~~ The resulting solution was charged  
onto a Protein G-Sepharose® column (~~manufactured by Pharmacia Co., Ltd.~~). After  
~~washed~~ washing the column with PBS, the ~~adsorbed~~ immunoglobulin G adsorbed was  
eluted with 0.1 M glycine-HCl hydrochloric acid buffer solution (pH 3.0). ~~The eluate,~~  
and the pH thereof was immediately ~~neutralized~~ adjusted to be neutral with 1.5 M Tris-  
HCl hydrochloric acid buffer (pH 8.7). After ~~dialyzing~~ the eluted protein  
~~fractions~~ fraction was dialyzed against PBS, the absorbance at 280 nm was measured ~~to~~  
~~determine the protein and its concentration was determined~~ ( $E^{1\%}_{1\text{cm}}$  13.5). ~~Anti~~ Horseradish  
peroxidase-labeled anti-OBM antibody ~~labeled with horseradish peroxidase~~ was prepared  
~~using a maleimide-activated peroxidase kit with~~ Maleimide Activated Peroxidase Kit  
(Pierce Co., Ltd.) ~~as follows.~~ That is, 80 µg of N-succinimide-S-acetyl  
~~thioacetic~~ acetylthioacetic acid was added to 1 mg of the purified antibody and  
~~reacted~~ allowed to react at room temperature for 30 minutes. ~~Five~~ 5 mg of hydroxylamine  
was added ~~to thereto for deacetylation, and then the resulting mixture to deacetylate the~~  
~~antibody.~~ The modified antibody was fractionated by using a polyacrylamide desalting  
column. The protein fractions ~~were~~ was mixed with 1 mg of maleimide- activated  
peroxidase and ~~reacted for one hour~~ allowed to react at room temperature ~~to obtain for 1~~  
hour, and then the enzyme-labeled antibody was obtained.

(2) — Capability of rabbit anti OBM polyclonal antibody to inhibit specific binding  
Inhibition of Specific Binding of the protein (OBM)-expressed Protein Expressed by the  
cDNA of the present invention Present Invention (OBM) or the natural type  
protein Natural-Type Protein of the p Present iInvention with to OCIF by Anti-OBM  
Rabbit Polyclonal Antibody

Purified 2 µg/ml of purified OBM (thioredoxin-OBM fused ion protein) obtained  
aeording to in accordance with the methods described in the Examples 15-(6) and 15-(7)  
and the 2 µg/ml of natural -type purified OCIF- binding protein of the Example 2-(4) were  
dissolved respectively in 0.1 M sodium carbonate buffer to a concentration of 2  
µg/ml hydrogencarbonate, respectively. An aliquot 100 µl of each solution was added  
100 µl per to each well respectively to of a 96-well immunoplate (manufactured by Nunc

Co.), Ltd. ~~The plate was allowed~~ and then left to stand at 4°C overnight at 4°C. 200 µl of 50% ~~Block Ace~~ BLOCKACE was added to each well and ~~the plate was allowed~~ left to stand at room temperature for ~~one~~ 1 hour. After washing ~~each well~~ wells three times with PBS containing 0.1% Polysorbate 20 (P20-PBS), ~~100~~ 200 µg/ml of rabbit-anti-OBM rabbit antibody solution which was dissolved in 25% ~~Block Ace~~ prepared BLOCKACE diluted with P20-PBS to a concentration, and 100 µl of 200 µg/ml the antibody solution or 100 µl of 25% ~~Block Ace~~ (containing no BLOCKACE without antibody) was added to each well, followed by incubation and incubated at 37°C for ~~one~~ 1 hour. Each well was washed. After washing wells three times with P20-PBS and charged with, 100 µl/well of a medium for the binding test solution experiment (P20-PBS containing 0.2% calf bovine serum albumin, 20 mM Hepes, and 0.1 mg/ml hHeparin) to which containing 20 ng/ml of the <sup>125</sup>I-labeled OCIF described in the Example 8-(3) was added thereto.

Alternatively, Furthermore, each well was charged with 100 µl/well of another medium for the binding test solution experiment containing 8 µg/ml of unlabeled OCIF in addition to 20 ng/ml of the <sup>125</sup>I-labeled OCIF was added to other wells. After incubating these immunoplates at 37°C for ~~one~~ 1 hour, ~~the each wells were~~ was washed six times with P20-PBS six times. The amount of <sup>125</sup>I in each well was measured by with a gamma counter. The results are shown in Figure Fig. 17. As shown in the figure, both the purified Fig. 17, neither OBM expressed using obtained by expressing the cDNA of the present invention and the protein that specifically binds subsequently purifying or the natural -type OCIF specifically binding protein of the present invention do not bind to, which specifically binds OCIF, bound the <sup>125</sup>I-labeled OCIF at all, when they were treated with the rabbit anti-OBM polyclonal antibody, whereas both proteins bound rabbit antibody. On the other hand, it was confirmed that both proteins bound to the <sup>125</sup>I-labeled OCIF when untreated not treated with the antibody. The binding of both proteins to <sup>125</sup>I-labeled said antibody. Furthermore, it was also revealed that bindings of both proteins to OCIF was confirmed to be clearly were specific, because those binding since the bindings were significantly inhibited by the addition of a 400-fold higher concentration of unlabelled OCIF (8 µg/ml). Based on From the above results described above, it was revealed that the rabbit anti-OBM rabbit polyclonal antibody recognizes both the OBM which is was the a protein expressed using by the cDNA of the present invention and the

natural-type OCIF- binding protein of the present invention, and it ~~inhibits the~~ inhibited specific binding of ~~these both~~ proteins with to OCIF.

<[Example 19]>

#### Cloning of hHuman OBM cDNA

##### (1) Preparation of mMouse OBM pPrimer

~~The~~ For screening of human OBM cDNA, a mouse OBM primers prepared ~~according to primer prepared in accordance with~~ the method of the ~~Examples (above~~ Example, OBM #3 and OBM#8) ~~described above, were used for screening of human~~ OBM cDNA #8 were used. ~~The Sequences sequence thereof are shown in the Sequence~~ Table, Sequence SEQ ID No. NO: 9 and No. 6, respectively. SEQ ID NO: 6.

##### (2) Acquisition of Human OBM cDNA Fragments by PCR

~~(2) — Isolation of A human OBM cDNA fragment by PCR Human OBM cDNA~~ fragments were ~~was~~ obtained by PCR method using the mouse OBM cDNA primers ~~prepared in (1) above and Human Lymph Node Marathon ready cDNA (Clontech Co.,~~ Ltd.) which was a human lymph node derived cDNA library as a template ~~and using~~ the mouse OBM cDNA primer prepared in the above (1).

The following are the conditions used for PCR—were shown as follows:

10-~~x~~ X EX Taq buffer (Takara Shuzo Co.,

22.0  $\mu$ l

2.5 mM dNTP 1.6  $\mu$ l

cDNA solution 1.0  $\mu$ l

EX Taq (Takara Shuzo Co., Ltd.) 0.2  $\mu$ l

Distilled ~~w~~ Water 14.8  $\mu$ l

40  $\mu$ M pPrimer OBM #3

0.2  $\mu$ l

40  $\mu$ M pPrimer OBM #8

0.2  $\mu$ l

~~These~~ After the above solutions were mixed together in a microfuge tube and pre- ~~incubated, PCR was conducted under the following conditions. A pretreatment was~~

~~carried out at 95°C for 2 minutes, followed by 40 cycles of a three-stage~~ then the cycle reaction consisted ~~ing of reactions at 95°C for 30 seconds, at 57°C for 30 seconds, and at 72°C for 2.5 minutes.~~ After the reaction ~~was repeated 40 times, and~~ the solution was incubated ~~at 72°C for an approx. 5 minutes at 72°C and a portion.~~ A subfraction of the solution ~~was subjected to reaction product and run through agarose by electrophoresis on an agarose gel.~~ A ~~detected an approximate 690 bp DNA fragment (about 690 bp)~~ amplified ~~by the~~ with the above mouse OBM cDNA primers ~~described above was detected.~~

(3) Purification of the human Human OBM cDNA fragment amplified Amplified by PCR and determination of the nucleotide sequence Nucleotide Sequence

The human OBM cDNA fragments ~~obtained in the Example 19-(2) was~~ were separated by agarose gel electrophoresis on an agarose gel and further then purified ~~using by use of a QIAEX® gel extraction kit (Qiagen QIAGEN Co., Ltd.).~~ PCR ~~was again performed using the~~ By use of the purified human OBM cDNA fragments ~~as a template and the templates,~~ PCR was conducted again by use of the above mouse OBM cDNA primers ~~described above, primer so as to produce~~ prepare a large quantity ~~amount of the human OBM cDNA fragment.~~ The DNA fragment ~~was~~ fragments which were then purified by ~~use of the QIAEX® gel extraction kit in the same manner as above.~~ The nucleotide sequence of the purified human OBM cDNA fragment was determined ~~using by use of a~~ Taq Dye Deoxy Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.) using OBM #3 ~~or~~ and OBM #8 (~~Sequence~~ SEQ ID No. ~~NO: 9 and No. 6~~ SEQ ID NO: 6, respectively) as a ~~primer~~ primers. ~~When compared with the~~ Comparing the nucleotide sequence of the human OBM cDNA fragment with the corresponding ~~are~~ part of the mouse OBM cDNA, ~~the nucleotide sequence of the human OBM cDNA fragment showed 80.7%~~ they share a homology with that of the mouse OBM cDNA ~~80.7%.~~

(4) Screening of a full length human for Full Length Human OBM cDNA by hybridization using the human Hybridization With Human OBM cDNA fragment (about Fragments With a Length of About 690 bp) as a ~~probe~~ Probes

~~A full length~~ The human OBM cDNA ~~was screened using the human OBM cDNA fragment (fragments, with a length of about 690 bp) that was, purified in the Example 19-(3) and~~ were labeled with [ $\alpha$ - $^{32}$ P] dCTP ~~using by use of a~~

~~MegaPrime~~MEGA PRIME DNA L-labeling kit (Amersham Co., Ltd.), and full length human OBM cDNA was screened. As an object to be screened, a Human Lymph Node 5' -STRETCH PLUS cDNA library (Clontech Co., the U.S.Ltd.A, USA) was screened using the DNA probe used. According to the manufacturer'sIn accordance with a protocol issued by the company, after *Escherichia coli* C600 Hfl was infected with the recombinant phage at 37°C for 15 minutes at 37°C. The infected, the *Escherichia coli* was added to an LB agar medium (1% trypton, 0.5% yeast extract, 1% NaCl, 0.7% agar) which was heated at 45°C. The LB agar was and poured onto an LB agar medium plate containing 1.5% agar. After overnight incubationculturing at 37°C, HyBond-  
HYBOND® N™ (Amersham Co., Ltd.) was placed to brought into intimate contact with the plate on whichhaving plaques were produced and storedformed thereon for about 3 minutes. According to a conventional methodThen, theis filter was treated withsubjected to an alkaline solutiondenaturation treatment in accordance with a commonly used method, neutralized, and dippedimmersed in a 2xSSC X SSC solution. The DNA was then immobilized ontofixed on the filter using theby UV CROSSLINKER (Stratagene Co., Ltd.). The resulting-obtained filter was dipped into was immersed in a Rapid-hyb buffer (Amersham Co.), Ltd. After pretreatment) and pretreated at 65°C for 15 minutes at 65°C, the. Thereafter, the filter was placed in Rapid-hyb transferred into the above buffer containing the above heat- denatured human OBM cDNA fragments (about 690 bp,  $5 \times 10^5$  cpm/ml) described above. Afterand allowed to hybridize at 65°C overnight hybridization at 65°C. After the reaction, the filter was washed with 2 x SSC, 1 x SSC, and 0.1 x SSC, each 0.1%-SDS-containing 0.1%-SDS2X SSC once, with 1X SSC once and with 0.1X SSC once in this order respectivelyturn at 65°C for 15 minutes at 65°C. SeveralThe obtained positive clones obtained were further purified by repeatingscreened two more times so as to purify the screening twiceclones. A clone possessing an insert (having about 2.2 kb) of insert was selected from the purified clonesout of these and was used in the following experiments. This purified phage was named  $\lambda$ -hOBM.  
AboutFrom the purified  $\lambda$ hOBM, about 10 µg of DNA was obtained from the purified  $\lambda$ -hOBM usingin accordance with a protocol of a QIAGEN® Lambda kit (QiagenQIAGEN Co., Ltd.) according to the manufacturer's protocol. TheAfter this DNA was digestedcleaved with a restriction enzyme SalI and subjected to electrophoresis

~~on an agarose gel to separate the~~ SalI, about 2.2 kb of hOBM insert cDNA (about 2.2 kb) ~~was separated by agarose electrophoresis. This DNA fragment, purified using the~~ use of a QIAEX® gel extraction kit (Qiagen QIAGEN Co., Ltd.), was digested ~~cleaved~~ with restriction enzyme SalI in advance and then inserted into dephosphorylated ~~plasmid pUC19 (MBI Co., Ltd.) which was previously digested with a restriction enzyme SalI and dephosphorylated, using~~ by use of a DNA ligation kit ver. 2 (Takara Shuzo Co., Ltd.). ~~Escherichia coli DH 5-α (Gibco BRL Co., Ltd.) was transformed with~~ by use of ~~the pUC19 containing the resulting~~ obtained ~~DNA fragment. The resulting~~ obtained ~~transformant was named pUC19hOBM. The~~ After proliferating the transformant was ~~grown and pUC19hOBM in which the,~~ about 2.2 kb of human -OBM cDNA (about 2.2 kb) was inserted and ~~plasmids were purified by~~ thereform in accordance with a ~~conventional~~ commonly used ~~method.~~

(5) Determination of nucleotide sequence ~~the Nucleotide Sequence of cDNA~~ Encoding the entire amino acid sequence ~~Full Length Amino Acid Sequence of a Human~~ OBM

The nucleotide sequence of the ~~resulting~~ human OBM cDNA obtained in Example 19 -(4) was determined ~~using the~~ by use of a Taq Dye-DeoxyDideoxy Terminator Cycle Sequencing FS kit (Paerkin Elmer Co., Ltd.). Specifically ~~That is,~~ the nucleotide sequence of the inserted fragment was determined ~~using~~ by use of pUC19hOBM as a template. ~~As primers, primers for the determination of the nucleotide sequence of the inserted fragment DNA in pUC19hOBM, M13 Primer M3 and 3, M13 Primer RV (manufactured by Takara Shuzo Co~~ TAKARA SHUZO CO., LTD.), and a synthetic primer, human OBM #8,8 designed based on the nucleotide sequence of the human OBM cDNA fragment (about 690 bp) were used. ~~The~~ as primers for determining ~~the nucleotide sequence of the inserted fragment DNA of pUC19. The sequences of the primers used, M13 Primer M3 and M13 Primer RV, are shown in SEQ ID NO: 4 and SEQ ID NO: 5, respectively shown as the Sequence ID No. 4 and No. 5. The amino acid sequence of human OBM deduced~~ estimated ~~from the nucleotide sequence of the human OBM cDNA is shown in the Sequence Table as Sequence~~ SEQ ID No. 11. The ~~NO: 11,~~ and the nucleotide sequence of the human OBM cDNA is shown as ~~in~~ Sequence ~~SEQ ID No.~~ NO: 12.



The obtained plasmid containing the human OBM cDNA and the obtained  
*Escherichia coli* which was transformed by the pUC19hOBM, which is the plasmid  
containing the resulting human OBM cDNA, was were deposited in with the National  
Institute of Bioscience and Human Technology, of the Agency of Industrial Science and  
Technology, on August 13, 1997 as of the Ministry of International Trade and Industry  
with the deposition No. number FERM BP-6058-6058 on August 13, 1997.

[Example 20]

Radioiodination  $^{125}\text{I}$  Labeling of OCIF with  $^{125}\text{I}$  and a Quantitative analysis

Determination of  $^{125}\text{I}$ -Labeled OCIF by ELISA

OCIF was  $^{125}\text{I}$ -labeled in accordance with  $^{125}\text{I}$  using the IODO-GEN Iodogen  
method. Twenty20  $\mu\text{l}$  of 2.5 mg/ml IODO-GEN Iodogen-chloroform solution was  
transferred to a 1.5 ml Eppendorf tube, and the chloroform was evaporated at 40°C,  
thereby providing a tube so as to prepare an Iodogen-coated with IODO-GEN tube.  
The After the tube was washed three times with 400  $\mu\text{l}$  of 0.5 M sodium phosphate  
buffer solution (Na-Pi; pH 7.0) three times, followed by the addition of 5  $\mu\text{l}$  of 0.5 M  
Na-Pi (with a pH of 7.0). To this tube was added. Immediately after 1.3  $\mu\text{l}$  (18.5 MBq)  
of Na- $^{125}\text{I}$  solution (Amersham Co., Ltd., NEZ-033H), immediately followed by was  
added to the addition of tube, 10  $\mu\text{l}$  of 1 mg/ml OCIF solution (monomer type or dimer  
type) was added. The mixture resulting solution was mixed in by means of a vortex mixer  
and allowed left to stand at room temperature for 30 seconds. This solution was  
transferred to a tube to which containing 80  $\mu\text{l}$  of 0.5 M Na-Pi (pH 7.0) solution  
containing (pH 7.0), which contained 10 mg/ml potassium iodide and 5  $\mu\text{l}$  of a  
phosphate buffered saline solution containing 5% bovine serum albumin (BSA-PBS)  
were previously added, and mixed. The solution was mixed, applied added to a spin  
column (1 ml, G-25 Sephadex® fine, manufactured by Pharmacia Co., Ltd.) which was  
equilibrated with BSA-PBS in advance, and centrifuged at 2,000 rpm for 5 minutes at  
2,000 rpm. Four After hundred 400  $\mu\text{l}$  of BSA-PBS was added to the a fractions eluted  
from the column. After mixing and the fraction was mixed, 2  $\mu\text{l}$  of the solution each  
fraction was used to measure sampled, and the radioactivity of the sample was measured  
by means of a gamma counter. The radiochemical purity of the prepared  $^{125}\text{I}$ -labeled  
OCIF solution obtained above was measured determined by counting measuring the

radioactivity of ~~fractions~~ a fraction precipitated by addition 10% trichloroacetic acid (TCA).

The OCIF biological activity of the  $^{125}\text{I}$ -labeled OCIF was measured according to thein accordance with a method described in WO 96/26217. TheFurther, the concentration of the  $^{125}\text{I}$ -labeled OCIF was measured using theby ELISA method as followsin the following manner. SpecificallyThat is, 100  $\mu\text{l}$  of 50 mM  $\text{NaHCO}_3$  (pH 9.6)-in which, having 2  $\mu\text{g/ml}$  of rabbit anti-OCIF polyclonal antibody described in the WO 96/26217 was dissolved to a concentration of 2  $\mu\text{g/ml}$ therein, was added to each well of a 96-well immunoplate (MaxiSorp<sup>TM</sup>, manufactured by Nunc Co.)-in the amount of 100  $\mu\text{l}$  per well. After these wells were allowed, Ltd., MaxiSorp) and left to stand at  $4^\circ\text{C}$  overnight-at  $4^\circ\text{C}$ ,. After this solution was removed. Then the wells were charged with a mixed aqueous-discarded, 200  $\mu\text{l}$  of mix-solution of Block Ace<sup>TM</sup>BLOCKACE (Snow Brand Milk Products Co., Ltd.) and a phosphate buffered saline solution-(mixing ratio = 25:75) (B-PBS) in the amount of 200  $\mu\text{l}$ /was added to each well. The plate was and then allowedleft to stand for two hours-at room temperature for 2 hours. After the solution was removeddiscarded, the each wells were was washed three times-with a phosphate buffered saline-solution containing 0.01% Polysorbate 80 (P-PBS) three times. NextThereafter, 100  $\mu\text{l}$  of B-PBS containing a  $^{125}\text{I}$ -labeled OCIF sample or theOCIF reference standard OCIF-was added in the amount of 100  $\mu\text{l}$ /to each well. The plate was then allowed and left to stand for two hours-at room temperature for 2 hours. After the solution was removeddiscarded, each well was washed six times-with 200  $\mu\text{l}$  of P-PBS six times. AThen, 100  $\mu\text{l}$  of diluted solution prepared by dilutingof peroxidase-labeled rabbit anti-OCIF rabbit polyclonal antibody within B-PBS was added in the amount of 100  $\mu\text{l}$ /to each well. The plate was allowed and left to stand for two hours-at room temperature for 2 hours. After the solution was removeddiscarded, the each wells were was washed six times-with 200  $\mu\text{l}$  of P-PBS six times. Then, a100  $\mu\text{l}$  of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added in the amount of 100  $\mu\text{l}$ /to each well. After being allowed and then left to stand at room temperature for 2- to 3 minutes. Thereafter, 100  $\mu\text{l}$  of a termination solution-(Stopping Reagent, (Scytek Co., Ltd.) was added to each well. AbsorbanceThe absorbance of each well at 450 nm was measured-at 450 nm-using by means of a microplate reader. The

concentration of the  $^{125}\text{I}$ -labeled OCIF was determined with from a calibration curve prepared using by use of the OCIF reference standard-OCIF.

<[Example 21]>

### **Expression of ~~the protein encoded~~ Protein Encoded by the cDNA of the ~~p~~Present ~~i~~Invention**

#### **(1) ~~Construction of hOBM e~~Expression ~~v~~Vector for aAnimal eCells**

pUChOBM was ~~digested~~cleaved with restriction enzyme SaHSalI, and a DNA fragment (about 2.2 kb) ~~were~~ DNA fragments were purified by 1% agarose gel electrophoresis on an 1% agarose gel. The ends of the DNA fragments were blunted using ~~a~~and blunt-ended with DNA ~~b~~Blunting kKit (Takara Shuzo Co., Ltd.) (blunted hOBMeDNA the resulting DNA fragment with smoothed terminals is called "smoothed hOBM cDNA fragment"). Expression plasmid pcDL-SR  $\alpha$ - $\alpha$ 296 (Molecular and Cellular Biology, Vol. 8, pp. 466- to 472 (1988)) was ~~digested~~cleaved with a restriction enzyme EcoRI, ~~blunted with~~and blunt-ended with the blunting kit ~~and ligated with the~~ blunted hOBM. The resulted expression plasmid was bound to the smoothed hOBM cDNA fragment using by use of a DNA ligation kit ver. 2. Using the ligation reaction solution, *Escherichia coli* DH- $\alpha$  was transformed with the ligation reaction. A plasmid in. From the resulting obtained ampicillin -resistant transformant was subjected to digestion with restriction enzyme to analyze the DNA restriction map and determine the DNA sequence. As a result, a strain clone, having a phOBM plasmid in which hOBM cDNA is inserted in the same with forward direction ~~effor~~ transcription as that direction of SR- $\alpha$  promoter  $\alpha$  promoter, was selected by analysis of DNA map obtained by restriction enzyme cleavage and determination of DNA sequences. The microorganism obtained strain clone was named DH5- $\alpha$ - $\alpha$ /phOBM.

#### **(2) ~~Expression of h~~Human OBM in COS-7 eCells**

~~Escherichia~~*E. coli*, DH5- $\alpha$ - $\alpha$ /phOBM, was cultured and the plasmid phOBM was purified using Qiafilter with QIA® Filter Plasmid Midi kKit (Qiagen QIAGEN Co., Ltd.). The phOBM was transfected using Lipofectamine into COS-7 cells in the each wells of a 6- well plate by use of lipofectamine, and the cells were cultured for two days in DMEM containing 10% fetal bovine serum for 2 days. The culture medium was replaced with cysteine-/methionine-free DMEM (manufactured by Dainippon Seiyaku Pharmaceutical

Co., Ltd.) ~~to which~~ containing 5% dialyzed fetal bovine serum ~~was added~~ (88  $\mu\text{l}$ /well). ~~The, and the~~ cells were ~~incubated~~ cultured for another 15 minutes. ~~Then, followed by~~ addition of 14  $\mu\text{l}$  of Express Protein Labeling Mix (NEN Co., Ltd., 10 mCi/ml) was added. After ~~four~~ the cells were cultured for 4 hours incubation, 200  $\mu\text{l}$  of DMEM containing 10% fetal bovine serum ~~was added to each well. The, and the~~ cells were cultured for ~~one~~ 1 hour and. ~~After the cells were~~ washed ~~twice~~ with PBS. ~~Then twice~~, 0.5 ml of  $\alpha$ -TSA buffer (10 mM Tris-HCl (pH 8.0) containing 0.14 M NaCl and 0.025%  $\text{NaN}_3$ , pH 8.0) containing 1% Triton X-100, 1% bovine hemoglobin, 10  $\mu\text{g}/\text{ml}$  leupeptin, 0.2 ~~TIU~~ TIU/ml aprotinin; and 1 mM PMSF ~~was added to each well, and the~~ mixtures ~~cells were allowed left to stand for one hour on ice for 1 hour. The~~ After the cells were ~~mixed~~ crushed by pipetting ~~and, the resulting lysate was~~ centrifuged at  $4^\circ\text{C}$  and 3,000 ~~X~~ g; for 10 minutes ~~at so~~ at  $4^\circ\text{C}$ , as to obtain supernatants a supernatant. Two hundred 200  $\mu\text{l}$  of a dilution buffer (TSA buffer containing 0.1% Triton X-100, 0.1% bovine hemoglobin, 10  $\mu\text{g}/\text{ml}$  leupeptin, 0.2 TIU/ml aprotinin; and 1 mM PMSF) was added to 100  $\mu\text{l}$  of the supernatant ~~from each well. The, and the~~ resulting mixtures ~~were~~ agitated at  $4^\circ\text{C}$  ~~for one hour~~ supernatant was shaken together with Protein A Sepharose® (50  $\mu\text{l}$ ) ~~and at~~ at  $4^\circ\text{C}$  for 1 hour. Thereafter, the solution was centrifuged at  $4^\circ\text{C}$ , 1,500 ~~X~~ g for ~~one~~ 1 minute ~~at so~~ at  $4^\circ\text{C}$ , as to collect supernatants a supernatant. Thereby, ~~thereby~~ removing the ~~a protein which non-specifically adsorbed~~ binding the Protein A Sepharose® was removed. OCIF (1  $\mu\text{g}$ ) was added to the ~~supernatants~~ supernatant, and the ~~mixtures~~ were ~~agitated~~ resulting supernatant was shaken at  $4^\circ\text{C}$  for ~~one~~ 1 hour ~~at so~~ at  $4^\circ\text{C}$  as to bind human-OBM and OCIF together. Then, ~~rabbit~~ anti-OCIF ~~rabbit~~ polyclonal antibody (50  $\mu\text{g}$ ) was added, ~~followed by agitation~~ and the resulting solution was shaken at  $4^\circ\text{C}$  for ~~one~~ 1 hour. Then, Protein A Sepharose® (10  $\mu\text{l}$ ) was added to the ~~resulting~~ solution; ~~followed by agitation~~ and the solution was then shaken at  $4^\circ\text{C}$  for ~~an additional~~ 1 hour. The ~~mixtures thus obtained~~ were resolution was centrifuged at  $4^\circ\text{C}$ , 1,500 ~~X~~ g for 1 minute ~~at 1,500 X g at~~ so as to collect precipitates a precipitated fraction. The ~~precipitates~~ were precipitate resulting from the centrifugation was washed ~~twice~~ with ~~the~~ dilution buffer twice, ~~twice~~ with a bovine hemoglobin- free dilution buffer twice, ~~once~~ with a TSA buffer once, and ~~once~~ with 50 mM Tris-HCl (pH 6.5) once. After ~~addition~~ washing,

of an SDS buffer containing 10%  $\beta$ -mercaptoethanol (0.125 M Tris-HCl, 4% dodecyl sodium dodecylsulfate, 20% glycerol, 0.002% bromophenol blue, pH 6.8), the mixture containing 10%  $\beta$ -mercaptoethanol was added to the precipitate. The precipitate was heated at 100°C for 5 minutes at 100°C, and it was subjected to SDS-PAGE (12.5% polyacrylamide gel, Daiichi Pure Chemical Kagaku Co., Ltd.). The gel was fixed and dried according to in accordance with a conventional commonly used method. After isotope, and the signals of isotopes from the fixed gel were enhanced amplified using by Amplify™ (Amersham Co.), the dried Ltd.). The fixed gel was subjected exposed to autoradiography at -80°C using Bio-Max BioMax® MR film (Kodak Co., Ltd.) at -80°C. The results are shown in Figure 18, which shows Fig. 8. As a result, it was revealed that the molecular weight of the protein encoded by the cDNA of the present invention is about 40,000.

<[Example 22]>

#### **Binding of the protein encoded Protein Encoded by the cDNA of the present Invention and to OCIF**

PhOBM, which was purified in the same manner as in the Example 21-(2), the purified phOBM was transfected into COS-7 cells in each well of a 24-well plate using Lipofectamine. After by the use of lipofectamine, and the cells were cultured for 2 to 3 days. Then, the cells were washed with serum-free DMEM. Two hundred, and 200  $\mu$ l of a culture medium for the binding test medium assay (serum-free DMEM to which containing 0.2% bovine serum albumin, 20 mM Hepes buffer solution, 0.1 mg/ml heparin, and 0.2%  $\text{NaN}_3$  were added), containing 20 ng/ml of  $^{125}\text{I}$ -labeled OCIF was added to the same wells. To the In addition, to other wells, 200  $\mu$ l of culture medium for the binding test medium the medium for binding assay, containing 8  $\mu$ g/ml of unlabeled OCIF in addition to 20 ng/ml of the  $^{125}\text{I}$ -labeled OCIF, was added. After incubation for one hour at 37°C in a so as to conduct following experiments. After culture in a  $\text{CO}_2$  incubator (5%  $\text{CO}_2$ ), the cells were washed twice with 500  $\mu$ l of a phosphate buffered saline solution containing 0.1 mg/ml of heparin. Then, 500  $\mu$ l of 0.1 N NaOH solution was added to each well and the plate was allowed at 37°C for 1 hour, the cells were washed twice with 500  $\mu$ l of phosphate buffered saline containing 0.1 mg/ml heparin. After washing, 500  $\mu$ l of 0.1 N NaOH solution was added to each well, and the wells

~~were then left to stand for 10 minutes at room temperature to dissolve the for 10 minutes so as to dissolve the cells. The radioactivity amount of  $^{125}\text{I}$  in the wells was measured by a gamma counter. As a result, as shown in Figure 19, in each well was measured by means of a gamma counter. As a result, it was confirmed that the  $^{125}\text{I}$ -labeled OCIF biounds only to the a cells transfected with phOBM. Moreover, as shown in Fig. 19. Further, it was also confirmed that the binding was significantly inhibited by adding 400-fold excess unlabelled OCIF (8  $\mu\text{g/ml}$ ). Based on the results described above, the protein, human OBM encoded by the cDNA in the phOBM was confirmed to specifically bind to OCIF on the surface of was significantly inhibited by addition of a 400-fold concentration of unlabeled OCIF (8  $\mu\text{g/ml}$ ). From these results, it was revealed that a human OBM protein, coded for by a cDNA on phOBM, specifically bound to OCIF on the surface of a COS-7 cells.~~

<

[Example-23>

23]

#### **Crosslinking of $^{125}\text{I}$ -labeled OCIF and Experiment of $^{125}\text{I}$ Labeled OCIF to Protein Encoded by the cDNA of the Present Invention**

~~To further analyze the characteristics of the protein encoded by the cDNA of the present invention, crosslinking of  $^{125}\text{I}$  labeled monomer type OCIF with the protein encoded by the cDNA of the present invention~~  
~~Crosslinking of  $^{125}\text{I}$ -labeled monomer type OCIF and the protein encoded by the cDNA of the present invention was carried out to further investigate the characteristics of the protein encoded by the cDNA of the present invention. After constructing expression vector phOBM and transfecting into COS-7 cells according to the method used in the~~ was conducted. That is, after expression vectors phOBM were prepared and transfected into COS-7 cells in accordance with the methods described in Examples 21-(1)1 and 21(2), 200 200  $\mu\text{l}$  of binding test medium the medium for the binding assay containing the  $^{125}\text{I}$ -labeled OCIF (25 ng/ml) was added to some wells. In addition, the medium for the binding assay, containing unlabeled OCIF of a 400-fold concentration in addition to the  $^{125}\text{I}$  labeled OCIF (25 ng/ml) described above, was added. The binding test medium to which unlabeled OCIF was added at a

400-fold concentration in addition to  $^{125}\text{I}$ -labeled OCIF was used for the other wells. After cultured for one hour at  $37^\circ\text{C}$  in a other wells. The cells were cultured in a  $\text{CO}_2$  incubator (5%  $\text{CO}_2$ ), the cells were washed twice with 500  $\mu\text{l}$  of phosphate buffered saline containing 0.1 mg/ml heparin. Five hundred  $\mu\text{l}$  of phosphate buffered saline in which at  $37^\circ\text{C}$  for 1 hour, and the cells were washed twice with 500  $\mu\text{l}$  of phosphate buffered saline containing 0.1 mg/ml of heparin. To these cells, 500  $\mu\text{l}$  of phosphate buffered saline containing 100  $\mu\text{g/ml}$  of a crosslinking agent (DSS: (dDisuccinimidyl suberate, manufactured by Pierce Co., Ltd.) was dissolved was added to, and the cells, followed by incubation incubated at  $0^\circ\text{C}$  for 10 minutes at for  $0^\circ\text{C}$  reaction. The After the cells in these wells were washed twice with 1 ml of ice-cold phosphate buffered saline. After an addition of cooled to  $0^\circ\text{C}$ , 100  $\mu\text{l}$  of 20 mM Hepes buffer solution containing 1% Triton X-100 (Wako Pure Chemicals Co. Chemical Industries, Ltd.), 2 mM PMSF (Pphenylmethylsulfonyl fluoride, Sigma Co., Ltd.), 10  $\mu\text{M}$  Ppepstatin (Wako Pure Chemicals Co. Industries, Ltd.), 10  $\mu\text{M}$  leupeptin (Wako Pure Chemicals Co. Chemical Industries, Ltd.), 10  $\mu\text{M}$  antipain (Wako Pure Chemicals Co. Chemical Industries, Ltd.) and 2 mM EDTA (Wako Pure Chemicals Co. Industries, Ltd.) were added to these cells, and the wells were allowed left to stand at room temperature for 30 minutes at room temperature minute so as to dissolve lyse the cells. These After 15  $\mu\text{l}$  of these samples (15  $\mu\text{l}$  aliquots) were treated with SDS under reducing nonreducing conditions according to in accordance with a conventional commonly used method and, the samples were subjected to electrophoresis with a gel for SDS- electrophoresis using (4- to 20% polyacrylamide gradient gel (, Daiichi Pure Chemical Kagaku Co., Ltd.). After the electrophoresis, the gel was dried and subjected exposed to autoradiography for 24 hours at  $-80^\circ\text{C}$  using BioMax® MS film Film (Kodak Co., Ltd.) with BioMax® MS Intensifying Amplifying Screen (Kodak Co. Co., Ltd.) and BioMax MS sensitization screen (Kodak Co.) at  $-80^\circ\text{C}$  for 24 hours. The film subjected to autoradiography was exposed films were developed according to in accordance with a conventional commonly used method. As a result, a protein band of having a molecular weight in the range of about 90,000–110,000, shown in Figure 20, to 110,000 was detected as shown in Fig. 20 by crosslinking of between  $^{125}\text{I}$ -labeled monomer type OCIF and the protein encoded by the cDNA of the present invention.

<[Example 24]>

### Expression of ~~sSecreted~~ory-form-Type ~~h~~Human OBM

#### (1) ~~Construction of sSecreted~~ory-formType ~~h~~Human OBM ~~eExpressi~~ng pPlasmid

A PCR reaction was carried out ~~using~~ by use of human OBM SF (~~Sequence-Table, Sequence~~SEQ ID No.~~NO:~~ 13) and mouse OBM #8 (~~Sequence-Table, Sequence~~SEQ ID No.~~NO:~~ 6) as primers and pUC19hOBM as a template. After the product was purified by agarose gel electrophoresis ~~on an agarose gel, the product was digested, it was cleaved~~ with restriction enzymes ~~SP1~~SpI and HindIII; and ~~further~~then purified by agarose gel electrophoresis ~~on an agarose gel~~ so as to obtain a purified fragment (0.27 kb) fragment. ~~Human OBMA~~ fragment of hOBM cDNA which was partially digested with ~~cleaved at only one site of~~ restriction enzyme DraI by partial cleavage of human OBM cDNA therewith, and DNA fragments digested with DraI at one site were purified by agarose gel electrophoresis ~~on an agarose gel. The, and the~~ purified fragment was further digested ~~cleaved~~ with a restriction enzyme HindIII. The 0.53 kb of DraI/HindIII fragment was purified by agarose gel electrophoresis ~~on an agarose gel. The, and the~~ purified fragment was ligated with ~~and~~ the 0.27 kb Sp1SpI/HindIII-fragment derived from fragment (0.27 kb) of the PCR-described above using ligation kit ver. 2 (Takara Shuzo Co.) PCR product together with ~~HindIII~~an SpI/EcoRI~~V~~ fragment (5.2 kb) of pSec TagA (~~Invitrogen~~Invitrogen Co.), Ltd. ) were subjected to ligation by use of a ligation kit ver. 2 (TAKARA SHUZO CO., LTD.), and Escherichia coli DH5- $\alpha$  was ~~transformed using~~ by use of the reaction product of ligation. Plasmids were purified by alkali-SDS method ~~from the resulting~~ obtained ampicillin -resistant transformants and digested with ~~clone by alkaline SDS method and cleaved by restriction enzymes so as to select a plasmid containing~~ having 0.27 kb and 0.53 kb- of fragments as inserts ~~inserted~~ in pSec TagA. This plasmid was confirmed ~~subjected to have a sequence encoding the secreted human OBM by sequencing using Tag-dye~~ deoxyterminator cycle sequencing by use of a Taq Dideoxy Terminator Cycle Sequencing FS kKit (Perkin Elmer Co., Ltd.), thereby confirming that the plasmid had sequences encoding secretory-type human OBM. ~~The~~After the plasmid was digested ~~cleaved~~ with ~~by~~ restriction enzymes NheI and XhoI ~~to prepare~~XhoI, a fragment (0.8 kb) corresponding to ~~the secreted~~secretory-type human OBM cDNA was collected by agarose gel electrophoresis ~~on an agarose gel~~. This



fragment was inserted into the NheI and XhoI fragment (10.4 kb) of an expression vector pCEP4 (Invitrogen Co., Ltd.) using by use of the ligation kit, and *Escherichia coli* DH5-~~α~~ were transformed using by use of the reaction product of the ligation. Plasmids were purified by alkali SDS method from the resulting obtained ampicillin - resistant transformants clones by alkaline SDS method and digested cleaved with by restriction enzymes so as to select a *Escherichia coli* clone having the a secretory-type human OBM expression plasmid for secreted form human OBM (pCEPshOBM) with a target structure. The *Escherichia coli* containing clone having the pCEPshOBM was cultured, and the pCEPshOBM was purified using Qiafilter plasmid midi kit by use of QIA® Filter Plasmid Midi Kit (Qiagen QIAGEN Co., Ltd.).

(2) Expression of sSecretedory-form Type OBM

293-EBNA cells were suspended in IMDM containing 10% FCS (IMDM-10% FCS), added into a seeded in a collagen-coated 24- well plate coated with collagen (manufactured by Sumitomo Bakelite Co., Ltd.) in a cell density an amount of  $2 \times 10^5$  cells/2 ml/well, and cultured overnight. The To the cells were transfected with, 1  $\mu$ g of pCEPshOBM or pCEP4 using was transfected by use of 4  $\mu$ l of Lipofectamine (Gibco Co.), Ltd. After, and the cells were cultured for two another 2 days in 0.5 ml of a serum-free IMDM or IMDM-10% FCS, the culture supernatants were collected thereby collecting a conditioned medium. Expression of the secreted secretory-type human OBM in the culture conditioned supernatant medium was detected as follows confirmed in the following manner. Sodium bicarbonate That is, sodium hydrogen carbonate was added to the culture conditioned supernatants medium to a final concentration of 0.1 M and the mixtures were added to a 96- well plate. The plate was allowed left to stand at 4°C overnight at 4°C, thereby and immobilizing the human OBM in the culture supernatants on the conditioned medium was solid-phased in a 96- well plate. The plate was blocked using Block Ace™ BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution four-fold diluted 4 times with PBS (B-PBS) was added to each well and allowed the plate was left to stand for two hours at room temperature for 2 hours to cause blocking. After adding 3-100 ng/ml of OCIF which was diluted with B-PBS was added to each well, the plate was allowed wells and left to stand at 37°C for two 2 hours at 37°C, followed by wash. After the plate was washed with PBS containing 0.05% Polysorbate 20 (P-PBS).

Then, 100  $\mu$ l of a peroxidase- labeled rabbit anti-OCIF polyclonal antibody described in WO 96/26217 which was diluted with B-PBS was added to each well. After allowing and left to stand at 37°C for two hours at 37°C, the wells were. After each well was washed six times with P-PBS. Then six times, 100  $\mu$ l of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added in the amount of 100  $\mu$ l per to each well and the mixture was allowed then left to stand at room temperature for about 10 minutes. The reaction was terminated by the addition of. Thereafter, 100  $\mu$ l of termination solution (Stopping Reagent, (Scytek Co., Ltd.) was added to each well. AbsorbanceThe absorbance of each well at 450 nm for each well was measured by means of a microplate reader. The results are shown in Figure 21, which indicates that the absorbance at 450 nm increased according to the concentration of the added OCIF in Fig. 21. In the plate in which having the solid-phased conditioned medium of the cells transfected with the pCEPshOBM was immobilized, absorption at 450 nm increased depending on the concentration of the OCIF added. On Meanwhile, in the case where the conditioned medium of the other hand, no increase in absorbance was seen in the wells in which the conditioned medium of the cells cells transfected with only with the vector pCEP4 was immobilized solid-phased, no increase in absorption was seen. Further, Figure Fig. 22 shows the results of an experiment wherein in which the proportion of the conditioned medium used for immobilizationsolid phasing was changed varied within a range of 5- to 90% in the presence of and a constant concentration of OCIF (50 ng/ml) was added. The absorbanceIn the plate having the solid-phased conditioned medium of the cells transfected with the pCEPshOBM, absorption at 450 nm increased according to the along with an increase in the proportion of the conditioned medium in the plate wherein the conditioned medium of the cells transfected with pCEPshOBM was immobilized added. Meanwhile, whereas no such increase in absorbance was seen in the plate wherein having the solid-phased conditioned medium of the cells transfected with the vector pCEP44, no increase in absorption was immobilized observed. From these results, it was confirmed that secretedory-form type human OBM is was produced expressed in the conditioned medium of the cells transfected with pCBPshOBM the pCEPshOBM.

<[Example 25]>

### **Expression of ~~f~~Thioredoxin-~~h~~Human OBM ~~f~~Fusion ~~p~~Protein (Trx-hOBM)**

#### **(1) Construction of a thioredoxinThioredoxin-hHuman OBM fFusion pProtein (Trx-hOBM) eExpression vVector**

Ten ~~10~~  $\mu\text{l}$  of 10X ExTaq buffer (Takara Shuzo Co., Ltd.), 8  $\mu\text{l}$  of 10 mM dNTP (Takara Shuzo Co., Ltd.), 77.5  $\mu\text{l}$  of sterilized distilled water, 2  $\mu\text{l}$  of ~~amp~~UC19hOBM aqueous solution of ~~p~~UC19hOBM (10 ng/ $\mu\text{l}$ ), 1  $\mu\text{l}$  of primer; mouse OBM #3 (SEQ ID NO: 9) (100 pmol/ $\mu\text{l}$ , Sequence Table, Sequence ID No. 9), 1  $\mu\text{l}$  of primer; hOBM SalR2 (SEQ ID NO: 14) (100 pmol/ $\mu\text{l}$ , Sequence Table, Sequence ID No. 14), and 0.5  $\mu\text{l}$  of ExTaq (5-u/ $\mu\text{l}$ ) (Takara Shuzo Co., Ltd.) were mixed and reacted (PCR) together in a ~~micro-centrifuge~~ microcentrifuge tube so as to cause a PCR reaction. After the reaction at ~~consisting of~~ 95°C for 5 minutes, at 50°C for ~~one~~ 1 second, at 55°C for ~~one~~ 1 minute, at 74°C for ~~one~~ 1 second, and at 72°C for 5 minutes, at the cycle reaction consisting of a reaction at 96°C for ~~one~~ 1 minute, at 50°C for ~~one~~ 1 second, at 55°C for ~~one~~ 1 minute, at 74°C for ~~one~~ 1 second, and at 72°C for 3 minutes, was repeated 25 times. From the total reaction mixture An approximately 750 bp DNA fragment (750 bp) was purified. The from the whole amount of reaction solution. After the purified DNA fragment (whole) was digested/cleaved with restriction enzymes SalI (Takara Shuzo Co. TAKARA SHUZO CO., LTD.) and BspHI (New England Bilabs Co. NEW ENGLAND BILABS CO., LTD.), and subjected to electrophoresis on a 1% agarose gel electrophoretic migration was carried out so as to obtain purified/purify an approximately 320 bp DNA fragment (Fragment 1, about 320 bp). The fragment was dissolved 1) and dissolve the fragment in 20  $\mu\text{l}$  of sterilized distilled water. In the same manner, Similarly, an approximately 450 bp DNA fragment (Ffragment-2, about 450 bp) obtained by digesting 2) which is a cleaved product of 4  $\mu\text{g}$  of pUC19hOBM with described in Example 19-(3) by a restriction enzymes BamHI, and BspHI (Takara Shuzo Co. TAKARA SHUZO CO., LTD.) and about 3.6 kb of DNA fragment (Fragment 3, about 3.6 kb/fragment 3), obtained by digesting which is a cleaved product of 2  $\mu\text{g}$  of pTrXFus (InVytrogen Co., Ltd.) withby a restriction enzymes BamHI, and SalI (Takara Shuzo Co. TAKARA SHUZO CO., LTD.) were respectively purified and then dissolved in 20  $\mu\text{l}$  of sterilized distilled water. The QIAEXHTo purify the DNA fragments, a QIAEXR

II gel extraction kit was used for purification of the DNA fragments. Fragments 1- Fragment 1, 2 and 3 were ligated combined by incubating at 16°C for 2.5 hours using use of a DNA ligation kit ver. 2 (Takara Shuzo Co. TAKARA SHUZO CO., LTD.) by keeping them at 16°C for 2.5 hours. Using the ligation reaction, Escherichia coli GI724 strain (Invitrogen Co., Ltd.) was transformed according to the using the ligation reaction solution, in accordance with a method described in the Instruction Manual of an instruction manual attached to a ThioFusion Expression System (Invitrogen Co., Ltd.). A microorganism strain with From the obtained ampicillin-resistant transformants, a clone, having a plasmid in which the hOBM cDNA fragment is fused in frame was bound to a thioredoxin gene in the same reading frame, was selected from the resulting ampicillin resistant transformants by analysis of DNA restriction map mapping obtained by digestion with restriction enzyme cleavage and by determination of DNA sequences. The microorganism strain thus obtained strain was named GI724/pTrxhOBM-25.

(2) Expression of Trx-hOBM OBM in Escherichia coli

A GI724/pTrxhOBM strain and a GI724 containing strain transformed with pTrxFus (GI724/pTrxFus) were respectively cultured six hours with shaking at 30/37°C for 6 hours in 2 ml of RMG-Amp medium (0.6% Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.05% NaCl, 0.1% NH<sub>4</sub>Cl, 2% casamino acid, 1% glycerol, 1 mM MgCl<sub>2</sub>, 100 µg/ml ampicillin, pH 7.4). The broth (0.5 ml) of the culture suspension was added to 50 ml of Induction medium (0.6% Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.05% NaCl, 0.1% NH<sub>4</sub>Cl, 0.2% casamino acid, 0.5% glucose, 1 mM MgCl<sub>2</sub>, 100 µg/ml ampicillin, pH 7.4) and cultured with shaking at 30°C. When OD<sub>600nm</sub> reached about 0.5, L-tryptophan was added so as to achieve a final concentration of 0.1 mg/ml, followed by culturing with when the value at OD<sub>600 nm</sub> became about 0.5, and the cells were further shaking-cultured at 30°C for an additional another 6 hours. The culture broth suspension was centrifuged at 3000/3,000 ×g so as to collect the cells, which were and then the collected cell was suspended in 12.5 ml of PBS. The suspension was subjected to an ultrasonic generator (Ultrasonics Co., Ltd.) so as to disrupt crush the cells. Then disrupt cells were the sample was centrifuged at 7000/7,000 ×g for 30 minutes so as to obtain a supernatant liquid as a collect a soluble protein fraction as a supernatant. Ten 10 µl of this soluble protein the solution fraction was subjected to SDS-PAGE (10% polyacrylamide (10%))

electrophoresis under reducing conditions. As a result, as shown in Figure Fig. 23, a protein band with having a molecular weight of 40,000 about 40,000, which was could not be seen in the soluble protein fraction of GI724/pTrxFus, was detected in the soluble protein fraction of GI724/pTrxhOBMpTrxOBM. Accordingly From the above results, it was confirmed that a thioredoxin-human OBM fusion protein (Trx-hOBM) of thioredoxin and human OBM) was expressed in the *Escherichia coli* clone.

### (3) Binding Ability of Trx-hOBM *Escherichia coli*-(3) — Binding capability of Trx-hOBM to OCIF

~~Binding of the expressed Trx-hOBM to OCIF~~ It was confirmed according to by the following experiment ~~that the expressed Trx-hOBM bound to OCIF. Anti~~ That is, 100  $\mu$ l of anti-thioredoxin antibody (Invitrogen Co., Ltd.) ~~which was diluted 5000-fold to be~~ 1/5,000 with 10 mM sodium hydrogen carbonate aqueous solution was added to each well of a 96- well immunoplate (Nunc Co., Ltd.), ~~in and the amount of 100  $\mu$ l per well.~~ After being allowed plate was left to stand at 4°C overnight at 4°C. After the liquidsolution in the each wellscell was discarded. Two, hundred 200  $\mu$ l of a solution preparedobtained by diluting Blook Ace™BLOCKACE (Snow Brand Milk Products Co., Ltd.) two-fold to be 1/2 with PBS (BA-PBS) was added to each well. After being allowed, and then the plate was left to stand for one hour at room temperature, for 1 hour. After the wells weresolution was discarded, each well was washed with P-PBS three times with P-PBS. The 100  $\mu$ l of the GI724/pTrxOBM-derived soluble protein fractions originating from fraction solution diluted stepwise with BA-PBS, and 100  $\mu$ l of the above-described GI724/pTrxhOBM or GI724/pTrxFus, each-derived soluble protein fraction solution diluted stepwise with BA-PBS in various concentrations, were added to each well in and the amount of 100  $\mu$ l. After being allowed plate was left to stand for two hours at room temperature, for 2 hours. After each well was washed with P-PBS three times with P-PBS and charged with, 100  $\mu$ l of OCIF (100 ng/ml) which was diluted with BA-PBS. After being allowed, was added to each well and the plate was left to stand for two hours at room temperature, for 2 hours. After each well was washed with P-PBS three times with P-PBS and charged with, 100  $\mu$ l of peroxidase-labeled anti-OCIF antibody (described in WO 96/26217) which was 26217, diluted to be 1/2,000-fold with BA-PBS.

After being allowed, was added to each well, and the plate was left to stand for two hours at room temperature, for 2 hours. After each well was washed six times with P-PBS and charged with six times, 100 µl of TMB solution. After being allowed was added to each well, and then the plate was left to stand at room temperature for about 10 minutes at room temperature. Thereafter, each well was charged with 100 µl of termination solution (Stopping Reagent). Absorbance was added to each well. The absorbance of each well at 450 nm was measured by means of a microplate reader. The results are shown in Figure Fig. 24. There was no difference in the absorbance was observed between absorbance resulted in the sample with presence and absence of the soluble protein fraction originating from GI724/pTrxFus-added thereto-derived soluble protein fraction solution and, while with the sample without the addition of this GI724/pTrxhOBM-derived soluble protein fraction. On the other hand solution, the absorbance increased depending on an increase in the samples to which the soluble protein fraction originating from GI724/pTrxhOBM was added in proportion to the concentration of the GI724/pTrxOBM derived soluble protein fraction solution. The Further, Fig. 25 shows the results of the other an experiment wherein in which the dilution rate of the soluble protein fraction solution to be added was maintained kept constant (1% concentration) while adding and OCIF (0-100 ng/ml) diluted stepwise with BA-PBS in different concentrations (0-100 ng/ml) are shown in Figure 25. was added. It can be seen that For the GI724/pTrxFus-derived soluble protein fraction solution, absorbance remained low at any concentrations regardless of the concentration of OCIF in samples using a soluble protein fraction originating from GI724/pTrxFus, whereas while for the GI724/pTrxhOBM-derived soluble protein fraction solution, absorbance increased in proportion to the OCIF concentration in the samples to which the soluble protein fraction originating from GI724/pTrxhOBM was added concentration-dependent manner. Based on these results, it was confirmed from this result that Trx-hOBM which is produced from GI724/pTrxhOBM has a capability of binding had an ability to bind OCIF.

(4) Large Scale-cultivation Culture of *Escherichia coli* which produces Producing Trx-hOBM

GI724/pTrxhOBM cells ~~were~~was spread on an RMG-Amp agar medium (0.6% Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.05% NaCl, 0.1% NH<sub>4</sub>Cl, 2% casamino acid, 1.5% agar, pH 7.4) using~~with~~ a platinum transfer 100p. The cells were~~loop and~~ cultured at 30°C overnight ~~at 30°C~~. The ~~cultured~~ cells were suspended in 10 ml of Induction medium. The, and every 5 ml of the suspension was added (~~5 ml for~~to each) ~~to~~of two 2-L Erlenmeyer conical flasks of 2L volume containing 500 ml of Induction medium, and the flasks were ~~shaking-~~shaking-cultured at 30°C ~~with shaking~~. When the OD<sub>600nm</sub> reached about 0.5, L-tryptophan was added so as to achieve a final concentration of 0.1 mg/ml. ~~Culturing with~~ when absorbance at OD<sub>600 nm</sub> became about 0.5, and the shaking culture at 30°C was continued for ~~six~~another 6 hours at 30°C. The culture ~~broth~~suspension was centrifuged at 3,000 X g for 20 minutes ~~at 3000 x g~~so as to collect cells and the collected cells, ~~which were then~~ suspended in 160 ml of PBS. The suspension was subjected to ~~an ultrasonic generator~~ultrasocination (Ultrasonics Co., Ltd.) so as to ~~disrupt~~crush the cells. ~~The supernatant liquid, and the cell lysate was then~~ centrifuged at 7,000 X g for 30 minutes ~~at 7000 x g~~so as to obtain collect a soluble protein fraction as a supernatant.

(5) Preparation of OCIF-Immobilized aAffinity eColumn

~~Two~~2 g of TSKgel AF-Tolresyl Toyopal~~TOYOPAL~~ 650 (~~Tosoh Corp~~TOSO CO., LTD.) and 40 ml of 1.0 M potassium phosphate buffer (pH 7.5) containing 35.0 mg of recombinant OCIF, ~~which was prepared according to the~~by a method described in WO 96/26217, were mixed. ~~The mixture was together and~~ gently shaken at 4°C overnight ~~at so 4°C~~as to effect cause a coupling reaction. ~~The reaction mixture was centrifuged to remove the supernatant.~~ To inactivate excessive active ~~residues~~residue, after a supernatant was removed by centrifugation, 40 ml of 0.1 M Tris-HCl buffer (pH 7.5) was added to ~~the~~a precipitated carrier, and the mixture was gently shaken at room temperature for ~~one~~1 hour. ~~The carrier in a column was washed with~~After 0.1 M glycine-HCl buffer (pH 3.3) containing 0.01% Polysorbate 80 and 0.2 M NaCl ~~and (pH 3.3) and a 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80 and 0.2 M NaCl.~~ ~~The carrier (pH 2.0) were passed through a column (in which the obtained gel was packed) so as to wash it,~~ the column was equilibrated by charging~~washed~~ twice with 10 mM sodium phosphate buffer (pH 7.4) containing 0.01% Polysorbate 80. 80 (pH 7.4) so as to equilibrate it.

(6) Purification of Trx-hOBM usingby OCIF-iImmobilized aAffinity eColumn

~~Unless otherwise indicated, purification~~Purification of Trx-hOBM was carried out at 4°C ~~unless otherwise stated.~~ The~~After the~~ above-mentioned OCIF-immobilized affinity carrier (10 ml) and the above-mentioned soluble protein fraction solution (120 ml) ~~prepareddescribed~~ in Example 25-(4) were mixed. ~~The together, the~~ mixture was gently shaken at 4°C overnight ~~at 4°C~~ in four 50 ml centrifuge tubes ~~usingby use of~~ a rotor. ~~An Econo-column™~~The carrier in the mixture was filled an EconoColumn (internal diameter: 1.5 cm, length: 15 cm, ~~manufactured by BioRad~~Bio-Rad Co., Ltd.) ~~was filled with the carrier in the mixture.~~ ~~The column was charged with~~ 300 ml of PBS containing 0.01% Polysorbate 80, 100 ml of 10 mM sodium-phosphate buffer (~~pH 7.0~~) containing 0.01% Polysorbate 80 and 2.0 M NaCl (~~pH 7.0~~), and 100 ml of 0.1 M glycine-HCl buffer (~~pH 3.3~~) containing 0.01% Polysorbate 80 and 0.2 M NaCl (~~pH 3.3~~) were passed through the column, in that orderturn, so as to wash the column. ~~NextThen,~~ ~~proteins adsorbed in the column were eluted with~~ 0.1 M sodium citrate buffer (~~pH 2.0~~) containing 0.01% Polysorbate 80 and 0.2 M NaCl (~~pH 2.0~~) was passed through the column so as to elute proteins adsorbed to the column. ~~The eluate was collected in-~~ 5 ml ~~portionseluates were fractionated.~~ ~~Each fraction thus collected was immediately neutralized with addition of~~ To the fractions, 10% volume of 2 M Tris-buffer solution (~~pH 8.0~~) was added so as to immediately neutralize the fractions. ~~PresenceThe presence~~ or absence of Trx-hOBM in the ~~eluted fractionseach fraction of the eluate~~ was ~~determined according to~~examined in accordance with the method ~~previously~~ described in Example 25-(3) ~~(the binding capability to OCIF).~~ The fractions containing Trx-hOBM were collected and purified further.

(7) Purification of Trx-hOBM by gGel fFiltration

About 25 ml of the Trx-hOBM fractions ~~obtaineddescribed~~ in Example 25-(6) was concentrated using a centrifuge to about 0.5 ml by ~~centrifuging usinguse of~~ a Centriplus R10 and a Centricon R10 (Amicon Co., Ltd.). ~~This~~The concentrated sample was ~~applied~~subjected to a Superose R12 HR 10/30 column (1.0 × 30 cm, Pharmacia Co., Ltd.) ~~previously~~equilibrated in advance with PBS containing 0.01% Polysorbate 80. ~~For the separation,~~The column was developed at a flow rate of 0.25 ml/min by using PBS containing 0.01% Polysorbate 80 ~~was used as a mobile phase at a flow rate of 0.25~~



~~ml/min. The eluate so as to fractionate every 0.25 ml of eluates from the column was collected in 0.25 ml portions. The Trx-hOBMOBM in the thus collected fractions was detected by the same method as previously described in the Example 25-(3) and SDS-PAGE. Fractions containing purified Trx-hOBM were collected and so as to measure the protein concentration of Trx-hOBM was determined OBM. The measurement of the protein concentration was carried out measured with DC-protein assay kit (Bio-Rad Co., Ltd.) using bovine serum albumin as a reference standard substance using DC-Protein assay kit (BioRad Co.).~~

<[Example 26]>

#### Osteoclast formation inducing activity of hOBM

#### Osteoclastogenesis Inducing Activity of OBM

phOBM and pcDL-SR- $\alpha$ 296 were respectively transfected into COS-7 cells using Lipofectamine by use of lipofectamine (Gibco Co., Ltd.), respectively. ~~The~~ After the cells were cultured for one day in DMEM containing 10% FCS for 1 day, they were trypsinized and seeded in a 24-well plate, plated on which glass cover slips (15 mm round shape, manufactured by Matsunami Co., Ltd.) in 24-well plates were seated, at a concentration of  $5 \times 10^4$  cells per/well, and then cultured for 2 another two days. The culture plate was washed once with PBS. ~~The cells were fixed with once and then~~ PBS containing 1% paraformaldehyde was added, and the cells were incubated at room temperature for 8 minutes so as to fix the cells on the glass cover slips. ~~The~~ After the plate on which the with fixed cells were attached was washed 6 times with PBS, then six times, 700  $\mu$ l of mouse spleen cells suspended at  $1 \times 10^6$ /ml in  $\alpha$ -MEM (containing  $10^{-8}$  M active form activated vitamin D<sub>3</sub>,  $10^{-7}$  M dexamethasone, and 10% fetal bovine serum) in an amount of  $1 \times 10^6$  cells/ml were added to each well. ~~Millieell~~ MILLICELL® PCF (Millipore Co., Ltd.) was set in on each well, and a 700 suspension  $\mu$ l of ST2 cells, suspended in the above-mentioned culture medium (in a concentration of  $4 \times 10^4$  cells/ml) were added, 700  $\mu$ l per well, into to the ~~Millieell~~ MILLICELL® PCF followed by incubation and cultured at 37°C for 6 days. After the culture that, the ~~Millieell~~ MILLICELL® PCF was removed, and the plate was washed once with PBS, and once. Then, the cells were fixed with for a minute by an acetone-ethanol solution (50:50) for one minute. Then, the and cells, exhibiting having tartaric acid- resistant acid

phosphatase activity (TRAP activity), which is a specific marker for osteoclast, were selectively stained using LEUKOCYTE ACID PHOSPHATASE by use of a leukocyte acid phosphatase kit (Sigma Co., Ltd.). As a result of microscopic observation, cells having TRAP-positive activity were not detected in the wells in which COS-7 cells transfected with the pcDL-SR  $\alpha 296$  were fixed. In contrast,  $\alpha 296$ , while 65  $\pm$  18 (n = 3, average  $\pm$  standard deviation, n=3) of TRAP positive cells were observed in the wells in which COS-7 cells transfected with phOBM were fixed. Moreover, expression of further, it was also confirmed that these TRAP positive cells expressed calcitonin receptor was confirmed by receptors, since the fact that cells showed specific binding to  $^{125}\text{I}$ -labeled salmon calcitonin (Amersham Co., Ltd.) specifically bound to these TRAP positive cells. Based on these findings, it has been proven that human OBM, which is a protein encoded by the cDNA of the present invention, has an activity to promote osteoclast formation-inducing activity.

<[Example 27]>

#### Osteoclast formation-inducing activity **Osteoclastogenesis Promoting Activities of Trx-hOBM and sSecretedory-formType hHuman OBM**

Mouse spleen cells were suspended in  $\alpha$ -MEM containing  $10^{-8}$  M active-vitamin D<sub>3</sub>,  $10^{-7}$  M dexamethasone, and 10% fetal bovine serum at a concentration of  $2 \times 10^6$  cells/ml. The, and 350  $\mu\text{l}$  of the suspension was added to each well of a 24 well plate in the amount of 350  $\mu\text{l}$  per well. Each well was then charged with After 350  $\mu\text{l}$  of a solution prepared by diluting purified Trx-hOBM (40 ng/ml) with the above-mentioned culture medium (40 ng/ml), 350  $\mu\text{l}$  of a solution prepared by 10-fold diluting a conditioned medium which was produced by culturing obtained when 293-EBNA cells, onto which transduced by pCEPshOBM or pCEP4 were transfected, cultured in a culture medium IMDM-10% FCS, to be 1/10 with the above-mentioned culture medium, or 350  $\mu\text{l}$  only of the above-mentioned culture medium. The Millicell PCF (Millipore alone was added, a MILLICELL® PCF (Millipore Co., Ltd.) was placed set on each well, to which and 600  $\mu\text{l}$  of ST2 cells which were suspended cell suspension in the above-mentioned culture medium (4  $\times$  at a concentration of  $4 \times 10^4$  cells/ml) were added to the Millicell® PCF. After the cell were cultured for

six days, the Millicell® PCF was removed. The plate was washed once with PBS and once. Then, after the cells were fixed for 1 minute by an acetone-ethanol solution (50:50) for one minute. Then, the cells exhibiting the activity of having tartaric acid resistant acid phosphatase activity (TRAP activity) were selectively stained using LEUKOCYTE ACID PHOSPHATASE by use of a leukocyte acid phosphatase kit (Sigma Co., Ltd.). The results of microscopic observation revealed that no Using a microscope, cells exhibiting the having TRAP activity were not detected in the wells to which not containing Trx-hOBM was added, whereas while  $115 \pm 19$  ( $n = 3$ , average  $\pm$  standard deviation,  $n = 3$ ) of TRAP- positive cells were observed in the wells to which containing Trx-hOBM was added. Similarly, while no cells exhibiting having TRAP activity were not detected in the wells to containing which the conditioned medium of 293pCEP4-EBNA cells transfected with pCEP4 had been added 293-EBNA, while  $125 \pm 23$  ( $n = 3$ , average  $\pm$  standard deviation,  $n = 3$ ) of TRAP positive cells were observed in the wells to containing which the conditioned medium of 293pCEPshOBM-EBNA cells transfected with pCEPshOBM had been added 293-EBNA. Moreover Furthermore, expression of calcitonin receptor it was also confirmed by the fact that these TRAP positive cells expressed calcitonin receptors, since the cells showed specific binding to  $^{125}$ I- labeled salmon calcitonin (Amersham Co., Ltd.) specifically binds to. From these TRAP positive cells. These results have proven, it was revealed that Trx-hOBM and secreted form hOBM exhibit secretory-type OBM had an activity to promote osteoclast formation-inducing activity.

<[Example 28]>

#### **Preparation of pPolyclonal aAntibody**

Mouse sOBM or human sOBM, which was used as an immunogen immunizing antigen, was prepared according to obtained in accordance with the above-mentioned method described above. Especially That is, mouse sOBM cDNA (cDNA (Sequence ID No. 18) encoding which encodes mouse sOBM (Sequence SEQ ID No. NO: 16) which does not have the having no membrane binding region of the mouse OBM due to absence of the site and lacking amino acids from between the N-terminal down to the 72nd end and amino acid 72 of mouse OBM; SEQ ID NO: 18) or human sOBM cDNA (cDNA (Sequence ID No. 19) encoding which encodes human sOBM (Sequence SEQ ID No. NO:

17) ~~which does not have the~~having no membrane binding region of human OBM due to ~~absence of the~~site and lacking amino acids ~~from region between the N- terminal down to the end and amino acid 71st amino acid of human OBM; SEQ ID NO: 19)~~was ligated, together with a Hind III/EcoRV fragment (5.2 kb) of the pSec TagA expression vector pSec-TagA (InVytrogen Co., Ltd.) ~~including the,~~containing nucleotide sequence ~~encoding~~coding a signal peptide of  $\kappa$ -chain of immunoglobulin, ~~together with~~and an EcoRI/PmaCI fragment (0.32 kb) of OBM cDNA, ~~using~~were subjected to ligation by use of a ligation kit ver. 2 (Takara Shuzo Co. TAKARA SHUZO CO., LTD.). *Escherichia coli* DH5  ~~$\alpha$ -was~~ $\alpha$  were transformed with the reaction product. ~~The plasmids~~Plasmids ~~were purified from the obtained from the resulting ampicillin -resistant strains were~~purified by the alkali clones by alkaline SDS method and digested with ~~and~~cleaved by restriction ~~enzyme~~enzymes so as to select a plasmid ~~with~~having 0.6 K~~b~~kb and 0.32 kb of fragments inserted ~~into~~in pSec TagA. ~~The sequence~~As a result of this ~~determining the~~sequences of the plasmid ~~was identified using the Dye deoxyterminator by use of Dye Terminator Cycle Sequencing FS kit (product of Perkin Elmer Co.), Ltd.~~As a result, it was confirmed that ~~this~~plasmid has a sequence ~~had~~sequences encoding mouse or human sOBM. After ~~The~~plasmid was digested ~~cleaved with~~by restriction enzymes NheI/XhoI, NheI and XhoI and then a fragment (1.0 kb) corresponding to secretionary form-type OBM cDNA was ~~recovered~~collected by agarose gel electrophoresis. This fragment was inserted into an NheI/XhoI fragment (10.4 kb) of the an expression vector pCEP4 (InVytrogen Co., Ltd.) ~~using~~by use of a ligation kit—, and *Escherichia coli* DH5  ~~$\alpha$ -was~~ $\alpha$  were transformed ~~using~~by use of the reaction product. Plasmids were purified ~~by the alkali SDS-~~from the resulting ~~obtained~~ampicillin -resistant strains. ~~Analyzing these plasmids by digesting with~~a clones by an alkaline SDS method and cleaved by restriction ~~enzyme,~~enzymes and ~~analyzed~~so as to select a *Escherichia coli* ~~possessing~~clone having a ~~secretion type~~secretory OBM expression plasmid (pCEP sOBM) ~~having with the~~objective target structure ~~was selected.~~ The *Escherichia coli* ~~strain~~clone having the pCEP sOBM was cultured, and the pCEP sOBM was purified ~~using~~by use of a Qiafilter plasmid midy kit QIA® Filter Plasmid Midi Kit (Qiagen QIAGEN Co., Ltd.). Next, 293-EBNA cells ~~were~~was suspended in IMDM containing 10% FCS (IMDM-10% FCS) ~~containing 10% FCS and~~plated ~~seeded onto~~in a 24- collagen-coated 24 well plate ~~coated with~~

collagen (product of Sumitomo Bakelite Co., Ltd.) at a cell density in an amount of  $2 \times 10^5$  cells/2 ml/well. After, and culturing overnight, To the cells were transformed with, 1  $\mu\text{g}$  of pCEP sOBM or pCEP4 using was transfected by use of 4  $\mu\text{l}$  of Lipofectamine (Gibco Co., Ltd.), and further the cells were cultured for two another 2 days in 0.5 ml of serum-free IMDM or IMDM-10% FCS, thereby collecting a conditioned medium. The culture supernatant was recovered. A cell line clones with high productivity on of recombinant mouse soluble OBM (msOBM) or human soluble OBM (hsOBM) was were screened as follows in the following manner. Sodium bicarbonate After sodium hydrogen carbonate was added to the culture supernatant which is assumed conditioned medium seemed to contain msOBM or hsOBM to a final concentration of 0.1 M. One, hundred 100  $\mu\text{l}$  of the culture supernatant conditioned medium was added to each well in of 96- well immunoplates (Nunc Co.) and allowed to stand, Ltd.) and the plate was left to stand at  $4^\circ\text{C}$  overnight at  $4^\circ\text{C}$ , thereby so as to solid-phase the msOBM or hsOBM in the culture supernatant was immobilized conditioned medium on each well. To each well Then, 200  $\mu\text{l}$  of Block Ace™ BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted four fold to 4 times with PBS (B-PBS) was added and the plates were allowed to stand for two hours to each well of the plate and the plate was left to stand at room temperature for 2 hours. After washing each well in the plates three times with PBS (P-PBS) containing 0.1% Polysorbate 20, 20 (P-PBS), 100  $\mu\text{l}$  of each recombinant OCIF (rOCIF) solution (3 diluted stepwise (0-100 ng/ml) diluted serially with P-PBS was added to each well in and the plates. The plates were allowed plate was left to stand at  $37^\circ\text{C}$  for two 2 hours at  $37^\circ\text{C}$ . After washing the plates three times with PBS, 100  $\mu\text{l}$  of a peroxidase- labeled anti-OCIF polyclonal antibody (WO 96/26217), diluted with B-PBS, was added to each well. After allowing and the plate was left to stand at  $37^\circ\text{C}$  for two 2 hours at  $37^\circ\text{C}$ , the wells were washed. After washing six times with P-PBS. Then, 100  $\mu\text{l}$  of TMB solution (TMB Soluble Reagent Agent, High Sensitivity, ScyTek Co., Ltd.) was added to each well in the plates and the plates were allowed left to stand at room temperature for about 10 minutes; subsequently the reaction was terminated by adding. Thereafter, 100  $\mu\text{l}$  of a stopping solution (Stopping Reagent, ScyTek Co.) to each (Scytek Co., Ltd.) was added to each well. The absorbance of each well. Absorbance at 450 nm of each well was measured

using by means of a microplate reader. It was confirmed that in the plate having the solid-phased protein derived from conditioned medium of the clone producing msOBM or hsOBM, the absorbance significantly increased remarkably in proportion to the concentration of the added OCIF. As in for the plates in which msOBM or hsOBM in the culture supernatant of the cell line clones producing msOBM or hsOBM was immobilized therein. The cell line that exhibited, clones indicating a high rate of increase in the absorbance were selected as a strain with high productivity highly producing clones thereof. Thus related 293 EBNA cells with high productivity Each of the highly producing clones of msOBM or hsOBM selected in the above mentioned manner were mass-cultured on a large scale in any use of IMDM medium containing 5% FCS, using as a medium in 25 T-flasks (T-225). After the cells reached grew to confluency, a 100 ml of fresh culture medium was added to each T-225 flask in the amount of 100 ml per 255 flask and the cells were further cultured for 3- or 4 days, to collect the culture supernatant and then a conditioned medium was collected. These procedures were repeated four By repeating this procedure 4 times to obtain, 10 L liters of the culture conditioned supernatant medium containing msOBM and 10 liters of the conditioned medium containing hsOBM were obtained. Purified msOBM (About 10 mg) or hsOBM ( of purified msOBM and about 12 mg) of purified hsOBM, which shows homogeneous band were uniform (molecular weight: 32 kDa) in terms of SDS-polyacrylamide gel-electrophoresis, were obtained from by carrying out purification on the culture supernatant by means of above-obtained conditioned medium with affinity chromatography using an OCIFr OCIF-immobilized column and gel filtration chromatography according to in accordance with the method described in Examples 25-(6) and 25(7). Each The thus-obtained purified preparations samples was were used as an antigen for immunization immunizing antigens. Each protein-antigen The obtained was antigens each were dissolved in phosphate buffered saline (PBS) to at a concentration of 200 µg/ml and emulsified then the solution was mixed with an equivalent volume amount of Freund's complete adjuvant so as to be emulsified. One 1 ml of the each emulsion was subcutaneously immunized administered to each of three Japanese white rabbits at intervals of about once every one week so as to immunize the rabbits. A booster injection An antibody titer was given measured, and when the antibody titer

reached a peak maximum, a booster was carried out. ~~Whole~~ 10 days after the booster, all blood was collected 10 days thereafter from all the rabbits. The ~~serum~~ Antiserum was diluted to two-fold times with a binding buffer for ~~p~~ Protein A sepharose Sepharose® chromatography (~~BioRad~~ Bio-Rad Co., Ltd.) and ~~applied~~ then added to a ~~p~~ Protein A column equilibrated with the ~~same~~ above buffer. After ~~washing~~ the column ~~extensively~~ was efficiently washed with the ~~same~~ above buffer, the ~~an~~ anti-sOBM antibody adsorbed to the column was eluted ~~with~~ by an elution buffer (~~BioRad~~ Bio-Rad Co., Ltd.) or 0.1 M glycine-HCl buffer, (pH ~~3.0-2.9~~ 2.9 to 3.0). ~~To~~ In order to immediately neutralize the ~~antibody-containing~~ eluate ~~immediately~~, the ~~eluate~~ eluted solution was fractionated ~~using~~ by use of a test tubes containing a small amount of 1.0 M Tris-HCl (pH 8.0). The ~~antibody~~ eluate was dialyzed ~~against~~ in PBS at 4°C overnight ~~at 4°C~~. The ~~antibody~~ content ~~amount of protein~~ in the antibody solution was measured ~~by~~ in accordance with the Lowry method using bovine IgG as a standard ~~protein~~. Thus, ~~about 10 mg of the~~ purified immunoglobulin (IgG) containing the polyclonal antibody of the present invention was obtained in an amount of about 10 mg per 1 ml of rabbit antiserum ~~was obtained~~.

<[Example 29]>

#### **Measurements of OBM and sOBM by ELISA Using Polyclonal Antibody**

Sandwich ELISAs, using the rabbit anti-hsOBM polyclonal antibody ~~A sandwich ELISA was constructed using the rabbit anti-human sOBM polyclonal antibody obtained in Example 28 as the a solid phase antibody and enzyme as an enzyme labeled antibody, were constructed.~~ Peroxidase ~~As enzyme labeling, peroxidase (POD) -labeled antibody labeling was prepared according to the~~ carried out in accordance with a method of Ishikawa *et al.* (Ishikawa *et al.*; J. Immunoassay, Vol. 4, 209-327, 1983 to 327, 1983). The ~~anti-human sOBM~~ hsOBM polyclonal antibody obtained in the Example 28 was dissolved in a 0.1 M NaHCO<sub>3</sub> ~~to solution at~~ a concentration of 2 µg/ml. ~~One hundred µg/ml, and 100 µl of the resulting solution was added to each well in of 96-well immunoplates (Nunc Co.), which was then allowed, Ltd.) and the plate was left to stand at room temperature overnight. Next~~ Then, 200 µl of 50% Block Ace™ BLOCKACE (Snow Brand Milk Products Co., Ltd.) was added to each well, and the plates ~~were allowed was left to stand for one hour at at room temperature. for 1 hour. The Each wells were was washed three times with PBS containing 0.1% Ppolysorbate 20 (washing~~

buffer)-Human three times. The purified human OBM, which was expressed according to the method of same manner as in Example 26 and was purified according to the method of same manner as in Example 2. The purified human OBM<sub>2</sub>, and the purified human sOBM, prepared obtained in example 28 were serially Example 28, was diluted stepwise with the first primary reaction buffer (0.2 M Tris-HCl buffer, pH 7.2, containing 40% BLock AceBLOCKACE and 0.1% Polysorbate 20 polysorbate 20, pH 7.2), respectively, and 100  $\mu$ l of the diluted solution was  $\mu$ l of each diluent were added to each well in. After the plates. The plates were allowed plate was left to stand at room temperature for two 2 hours, and each well was washed three times with the above-mentioned washing buffer three times. Subsequently, 100  $\mu$ l of POD- labeled anti-human sOBM polyclonal antibody, diluted 1000 fold 1,000 times with the second secondary reaction buffer (0.1 M Tris-HCl buffer, pH 7.2, containing 25% BLock AceBLOCKACE and 0.1% Polysorbate 20 polysorbate 20, pH 7.2) was added to each well in and the plates. After the plates were allowed plate was left to stand at room temperature for two 2 hours, Each well was washed three times with the washing buffer three times. Next, 100  $\mu$ l of enzyme substrate solution (TMB, ScyTek Co., Ltd.) was added to each well in the plates, and the plates were allowed was left to stand at room temperature for 10 minutes, followed by the addition of. 100  $\mu$ l of a reaction termination stopping solution (Stopping reagent, ScyTek Co., Ltd.) was added to each well so as to stop the enzyme reaction. The absorbance at 450 nm of each well was measured using by use of a microplate reader. The results are shown in Figure Fig. 26. The sandwich ELISA, using a the rabbit anti-human sOBM polyclonal antibody recognized, almost equally detected both human sOBM (molecular weight; about 32 kDa) and human OBM (molecular weight; about 40 kDa), with a and measurement sensitivity of was about  $12.5 \times 10^{-3}$  pmol/ml (human OBM: about 500 pg/ml; for human sOBM: OBM, about 400 pg/ml for human sOBM). The measurement It was revealed that measurements of mouse sOBM and mouse OBM by ELISA using the rabbit anti-mouse sOBM polyclonal antibody obtained in the Example 28 was able to could be carried out made in the same manner. It was confirmed that an extremely as described above, measurement sensitivity in measuring mouse OBM or mouse sOBM was similar with that in human OBM or human sOBM, and a very small amount of mouse sOBM or mouse



~~OBM can be measured with almost the same sensitivity as described above could be measured.~~

~~As mentioned above, the~~

~~As described above, since the present anti-human sOBM polyclonal antibody of the present invention prepared in the Example 28 can equally recognize both the polyclonal antibody obtained in Example 28 recognized both human sOBM and human OBM antigens. Therefore, the antibody was as antigen equally, it was named an anti-human OBM/-human OBM/sOBM polyclonal antibody. Meanwhile, since the anti-mouse sOBM polyclonal antibody. Similarly, the anti-mouse antibody obtained in Example 28 recognized both mouse sOBM and mouse OBM as antigen equally, it was named an anti-mouse OBM/sOBM polyclonal antibody prepared in the Example 28 can equally recognize both the mouse sOBM and mouse OBM antigens. This antibody was therefore named anti-mouse OBM/sOBM polyclonal antibody.~~

~~<Example 30>~~

~~Preparation of monoclonal antibody antibody.~~

~~[Example 30]~~

### **Preparation of Monoclonal Antibody**

~~The purified human sOBM prepared obtained in the Example 28 was used as the antigen for immunization an immunizing antigen. The purified human sOBM was dissolved in physiologicalphosphate buffered saline solution to at a concentration of 10 µg/ml and emulsified by mixing with an equivalent volume of Freund's complete adjuvant µg/ml. To the prepared human sOBM solution, an equal amount of Freund's complete adjuvant was added so as to emulsify it. The emulsion was intraperitoneally Thereafter, 200 µl of the antigen was administered to BALB into the abdominal cavity of each Balb/c micemouse at an interval of one week for a desetotal of 200 µl three times, once a week, so as to immunize the mice. Next Then, the equivalent volume of the Freund's complete adjuvant was added to a physiological saline solution containing 5 µg/ml of the human sOBM, an equal amount of Freund's incomplete adjuvant was added so as to fully emulsify it, and 200 µl of the mixture was sufficiently emulsified. This emulsion was injected intraperitoneally to BALB administered to each~~

~~of the above Balb/c mice at a dose of 200  $\mu$ l, once a week intervals for a total of four weeks for immunization times so as to further immunize the mice. One~~ After the passage of one week ~~after from~~ the fourth additional immunization, 100  $\mu$ l of ~~aphosphate~~ physiological buffered saline solution containing 10  $\mu$ g/ml of the human sOBM was intravenously ~~parenterally~~ administered to each of the ~~BALB~~ Balb/c mice as a ~~for~~ booster. ~~After three days~~ On the 3<sup>rd</sup> day after the final immunization, the spleen was ~~extracted removed~~, and spleen cells were separated. ~~The spleen cells were and~~ fused with mouse myeloma cells, P3x63-Ag ~~AG~~8.653 according to ~~in accordance with a~~ conventional ~~known~~ method (Koehler, G. and Milstein, C., Nature, 256, 495 (1975)). ~~The suspended fused cells were~~ After completion of the fusion, the cell suspension was cultured for 10 days in a ~~an~~ HAT medium containing hypoxanthine, aminopterin, and thymidine for 10 days. After the myeloma cells ~~were dead~~ perished and hybridomas appeared, the HAT-medium was replaced with an HT medium obtained by removing aminopterin ~~free from the~~ HAT medium, and the cell culture was continued.

<[Example 31]>

#### **Selection of and Cloning of Hybridoma**

~~Since the appearance of the hybridoma and cloning~~ Appearance of hybridomas was recognized 10 days ~~after seen on the 10<sup>th</sup> day from the start of the cell fusion and~~ culturing in Example 30. ~~Monoclonal antibodies~~ 30, a high affinity antibody recognizing the human sOBM with high affinity and hybridomas ~~and hybridoma~~ producing these antibodies were selected according to ~~in the means of~~ the following procedure using the improved solid phase ELISA ~~which is described below. In addition~~ Further, to select the ~~an~~ anti-OBM monoclonal antibody which ~~recognizes~~ recognizing both of human sOBM and mouse sOBM, the mouse sOBM ~~prepared~~ obtained in the Example 27 was used ~~in addition to~~ as well as human sOBM was used as the ~~an~~ antigen for in the solid phase ELISA. The ~~human~~ Human sOBM and mouse sOBM were ~~each~~ respectively was dissolved in a 0.1 M sodium ~~bicarbonate~~ hydrogen carbonate solution at a concentration of 5  $\mu$ g/ml. Fifty ~~ml~~, and 50  $\mu$ l of each antigen solution was added to each well in of a 96-well immunoplates (Nunc Co.), Ltd. ~~The plates were allowed~~, and the plate was left to stand at 4°C overnight so as to ~~immobilize~~ attach the antigens. The antigen solution in each well was discarded. ~~Each well was then filled with, and~~ 200  $\mu$ l of 50% Block

Ace™BLOCKACE (Snow Brand Milk Products Co., Ltd.) ~~and allowed~~ was added to each well. The plate was left to stand at room temperature for ~~one~~ 1 hour so as to cause blocking. After each well was washed with a phosphate buffered saline solution (PBS-P) containing 0.1% ~~P~~polysorbate 20, 40  $\mu$ l of ~~ealf~~bovine serum (~~Hielone Inc~~Hyclone Co., Ltd.) was added to each well. ~~Subsequently~~ Then, 10  $\mu$ l of each hybridoma culture ~~supernatant~~ conditioned medium was added to each well and ~~each the well~~ the well plate was ~~incubated~~ left to stand under a serum concentration of 80% at room temperature for ~~two~~ 2 hours in the presence of 80% calf serum so as to cause reaction. ~~The~~ An object of the solid phase ELISA in the presence of 80% calf serum is to select ~~a hybridoma which produce~~ an antibody ~~which can detect~~ capable of binding to a very small amount of human sOBM or mouse sOBM even in ~~a solution containing high concentration~~ the presence of protein and ~~in the presence of an immunoreaction interfering~~ a serum-derived immune reaction inhibiting substance derived from serum in high concentration, i.e. that is, to select a hybridoma ~~which can produce~~ producing an antibody ~~with a~~ having high affinity for human sOBM or mouse sOBM. After completion of the reaction at room temperature for ~~two~~ 2 hours, the plates ~~were~~ was washed with PBS-P, and ~~subsequently~~, 50  $\mu$ l of diluent of peroxidase- labeled anti-mouse IgG (KPL Co., LTD.) diluted ~~5000 fold~~ to 5,000 times with a physiological saline solution containing 25% ~~Block Ace~~ BLOCKACE was added to each well. ~~After, and the reaction~~ plate was left to stand at room temperature for ~~two~~ 2 hours, so as to cause a reaction. After the plate was washed with PBS-P three times ~~with PBS-P~~. After the addition of, 50  $\mu$ l of an enzyme substrate solution (TMB, ScyTek Co., Ltd.) was added to each well, ~~the reaction was continued~~ and left to stand at room temperature for ~~five~~ 5 minutes. ~~The enzymatic reaction was~~ stopped by the addition of Then, 50  $\mu$ l of a ~~termination solution~~ (reaction stopping reagent (Stopping Reagent, ScyTek Co., Ltd.) was added so as to terminate the enzyme reaction. Hybridomas ~~which produce antibodies recognizing human sOBM or mouse sOBM were selected by measuring~~ The absorbance at 450 nm of each well using was measured by use of a microplate reader (~~Immune Reader~~ IMMUNOREADER NJ2000™, 2000, Nippon InterMed Co.), Ltd. ~~Hybridomas~~) so as to select a hybridoma producing antibodies exhibiting an antibody which recognizes human sOBM or mouse sOBM. The hybridomas showing particularly high absorbance (OD<sub>450nm</sub>) were selected.

~~Cloning of these hybridomas and repeatedly cloned 3 to 5 times~~ by a limiting dilution method ~~was repeated 3 so as to 5 times to establish stable~~ established hybridomas producing antibody stably. ~~Hybridomas exhibiting particularly high~~ Out of the obtained hybridomas, hybridomas having higher antibody productivity were selected ~~among the established~~ antibody-producing hybridoma clones.

<[Example 32]>

#### **Production and pPurification of mMonoclonal aAntibody**

The ~~antibody-producing hybridomas~~ antibodies obtained in the Example 31, i.e. ~~high affinity antibody producing that is, the~~ hybridoma producing an antibody which recognizes human sOBM with high affinity and the hybridoma producing the antibody which ~~produces an antibody showing~~ has a cross-reactivity ~~to the~~ with mouse sOBM were cultured, ~~respectively, and~~ Each hybridoma was implanted ~~intraperitoneally to BALB in~~ the abdominal cavity of a Balb/c-mice-based mouse which had been given pristane (Aldrich Chemical Co., Ltd.) about a week before, in an amount of  $1 \times 10^6$  cells per /mouse) ~~to which pristan (Aldorich Co.) was administered one week previously~~. After about 2- or 3 weeks, accumulated ascites were collected. ~~The~~ was sampled so as to obtain ascites containing the monoclonal antibody, which recognizes antibody recognized human sOBM ~~of the present invention or both the~~ monoclonal antibody recognizing human sOBM and mouse sOBM. Purified monoclonal antibodies were obtained from the ascites using Protein A column (Pharmacia Co., Ltd.) chromatography in the ascites, was ~~purified according to the purification~~ accordance with the method of ~~for purifying an anti-~~ OBM/sOBM polyclonal antibodies using a Protein A column antibody described in the Example 28. ~~The purified monoclonal antibody was thus obtained from the ascites by Protein A column chromatography (Pharmacia Co.).~~

<[Example 33]>

#### **Antigen specificity of monoclonal antibody**

#### **Antigenic Specificity of the Monoclonal Antibody**

The antigenic specificities of ~~a monoclonal antibody~~ antibodies, which specifically recognizes human sOBM, and ~~the of monoclonal antibody~~ antibodies, ~~exhibiting~~ having cross-reactivity ~~to both the~~ with human sOBM and mouse sOBM ~~was~~ investigated, were examined using human sOBM, human-intact human OBM having a

membrane binding regionsite, mouse sOBM, and ~~mouse-intact sOBM~~ mouse OBM having a membrane binding regionsite as antigens. ~~More than thirty kinds~~ Although over 30 types of monoclonal antibodyies were obtained. ~~The results on several of~~ representative monoclonal antibodies are shown in Table 1. As a result, it was ~~found~~ revealed that most of anti-human sOBM monoclonal antibodies which specifically recognized human sOBM ~~also recognize the human~~ recognized even intact human OBM having a membrane binding region, ~~but~~ site and did not ~~there~~ recognize mouse ~~OBMs~~ OBM and ~~the mouse-intact mouse OBM which has~~ having a membrane binding regionsite. ~~On the other hand~~

Meanwhile, ~~it was found that only~~ a few monoclonal antibodies recognizing both the of human sOBM and mouse sOBM were also obtained and it was found that these antibodies ~~exhibit~~ had cross-reactivity to both the with human OBM and mouse OBM. These results ~~show~~ indicate that ~~there is a common antigen recognizing sites, namely a common epitopes, in both the human OBM and mouse OBM~~ had a common antigen recognition site, i.e., epitope. ~~Based on the fact that the~~ Since an anti-human sOBM monoclonal antibody prepared ~~using the~~ by use of human sOBM as an ~~immune~~ antigen also equally recognized human OBM ~~having, which was a membrane binding region~~. ~~Anti-human sOBM intact protein, the~~ monoclonal antibody was named the anti-human OBM/sOBM monoclonal antibody.

TABLE 1

Antibody	Antigen			
	hsOBM	hoBM	MsOBM	mOBM
H-OBM-1	+	+	-	-
H-OBM-2	+	+	-	-
H-OBM-3	+	+	-	-
H-OBM-4	+	+	-	-
H-OBM-5	+	+	-	-
H-OBM-6	+	+	-	-
H-OBM-7	+	+	-	-
H-OBM-8	+	+	-	-
H-OBM-9	+	+	+	+
H-OBM-10	+	+	-	-
H-OBM-11	+	+	-	-
H-OBM-12	+	+	-	-
H-OBM-13	+	+	+	+
H-OBM-14	+	+	-	-

Table 1

Antibody	Antigen			
	hsOBM	hOBM	msOBM	mOBM
H-OBM 1	+	+	-	-
H-OBM 2	+	+	-	-
H-OBM 3	+	+	-	-
H-OBM 4	+	+	-	-
H-OBM 5	+	+	-	-
H-OBM 6	+	+	-	-
H-OBM 7	+	+	-	-
H-OBM 8	+	+	-	-
H-OBM 9	+	+	+	+
H-OBM 10	+	+	-	-
H-OBM 11	+	+	-	-
H-OBM 12	+	+	-	-
H-OBM 13	+	+	+	+
H-OBM 14	+	+	-	-

(hsOBM: human soluble OBM, hOBM: human membrane ~~bonding type~~binding OBM, msOBM: mouse soluble OBM, mOBM: ~~humanmouse~~ membrane ~~bonding type~~binding OBM)

<[Example 34]>

#### Determination of

#### Tests of eClass and sSubclass of mMonoclonal aAntibody

The class and subclass of the monoclonal antibody of the present invention were determined by ~~the immunoglobulin class and subclass analysis kit~~use of the Immunoglobulin Class/Subclass Analytical Kit (Amersham Co., Ltd.)~~according to the~~. The tests were conducted in accordance with a protocol ~~indicated~~provided in the kit. The results ~~enof~~of representative monoclonal antibodies are shown in Table 2. ~~As shown in Table 2, the~~The majority of anti-human OBM/sOBM monoclonal antibodies ~~were~~had IgG<sub>1</sub>, ~~the others were~~and some antibodies having IgG<sub>2a</sub> ~~and/or~~ IgG<sub>2b</sub> were also found. ~~Light chains for~~Further, all of the antibodies ~~were~~~~κ chain~~had κ chain as a light chain.

TABLE 2

Antibody	IgG <sub>1</sub>	IgG <sub>2a</sub>	IgG <sub>2b</sub>	IgG <sub>3</sub>	IgA	κ
H-OBM 8	-	+	-	-	-	+
H-OBM 9	+	-	-	-	-	+
H-OBM 10	+	-	-	-	-	+
H-OBM 11	+	-	-	-	-	+
H-OBM 12	-	-	+	-	-	+

H-OBM 13	+	-	-	-	-	+
H-OBM 14	+	-	-	-	-	+

Table 2

<

Antibody	IgG <sub>1</sub>	IgG <sub>2a</sub>	IgG <sub>2b</sub>	IgG <sub>3</sub>	IgA	κ
H-OBM 8	-	+	-	-	-	+
H-OBM 9	+	-	-	-	-	+
H-OBM 10	+	-	-	-	-	+
H-OBM 11	+	-	-	-	-	+
H-OBM 12	-	-	+	-	-	+
H-OBM 13	+	-	-	-	-	+
H-OBM 14	+	-	-	-	-	+

[Example 35>]

# **Measurement of the dissociation constant ( $K_d$ ) Dissociation Constant ( $K_D$ value) of monoclonal antibody for the Monoclonal Antibodies**

The dissociation constant of the constants for monoclonal antibodies was measured according to in accordance with a known method (Betrand Friguet *et al.*: Journal of Immunological Methods, 77, 305- to 319, 1986). That is, the purified antibody obtained in the-Example 32 was diluted at 5 ng/ml with 0.4 M Tris-HCl buffer (a primary buffer, pH 7.4) containing 40% Block Ace BLOCKACE and 0.1% Ppolysorbate 20 to give a concentration of 5 ng/ml. The solution was mixed with (pH 7.4, primary buffer) and an equivalent volume amount of a diluted solution diluent of the purified human soluble OBM (hsOBM) obtained in Example 28 in 28, prepared with the primary buffer with a at stepwise-concentration range of from 6.25 ng/ml to 10 µg/ml. The mixture was allowed, was added and the solution was left to stand at 4°C for 15 hours at 4°C as to bind the hsOBM to the monoclonal antibody to hsOBM. After 15 hours, the antibody not bound unbound to the hsOBM (10 µg/ml, 100 µl/well) hsOBM was measured using an immobilized by solid phase ELISA with solid-phased hsOBM (10 µg/ml, 100 µl/well) so as to calculate the dissociation constant of the monoclonal antibody to the hsOBM. In addition, Further, the affinity to for msOBM of an antibody, which is a monoclonal antibody for the hsOBM and also exhibits the antibodies, having cross-reactivity to with mouse soluble OBM (msOBM) and hsOBM, was also measured according to the same method except for by using msOBM instead of in place of hsOBM

at the above-mentioned method. Particularly, the hsOBM. Dissociation constant results of particular antibodies, which exhibit had high affinity to for each antigen of the antigens and are were useful for in enzymatic immunoassay and, binding assay and such, are shown in Table 3.

Table 3

Antibody	Subclass	Antigen	Dissociation constant Kd (M)
H-OBM 1	IgG <sub>1</sub> (κ)	hsOBM	$1 \times 10^{-11} < k_d < 1 \times 10^{-10}$
H-OBM 4	IgG <sub>1</sub> (κ)	hsOBM	$1 \times 10^{-11} < k_d < 1 \times 10^{-10}$
H-OBM 9	IgG <sub>1</sub> (κ)	hsOBM	$1 \times 10^{-9} < k_d < 1 \times 10^{-8}$
H-OBM 9	IgG <sub>1</sub> (κ)	msOBM	$1 \times 10^{-8} < k_d < 1 \times 10^{-7}$

Antibody	Subclass	Antigen	Dissociation Constant Kd (M)
H-OBM 1	IgG <sub>1</sub> (κ)	hsOBM	$1 \times 10^{-11} < K_d < 1 \times 10^{-10}$
H-OBM 4	IgG <sub>1</sub> (κ)	hsOBM	$1 \times 10^{-11} < K_d < 1 \times 10^{-10}$
H-OBM 9	IgG <sub>1</sub> (κ)	hsOBM	$1 \times 10^{-9} < K_d < 1 \times 10^{-8}$
H-OBM 9	IgG <sub>1</sub> (κ)	msOBM	$1 \times 10^{-8} < K_d < 1 \times 10^{-7}$

As a result, the dissociation constants (Kd) of it was found that H-OBM 1 and H-OBM 4 which are the were specific antibodies specific to for human soluble OBM (hsOBM) were in showed a dissociation constant on the order of  $10^{-11}$  M, indicating th that they had very high affinity to for hsOBM. On the other hand Meanwhile, the Kd value of the antibody H-OBM 9 which recognizes was an antibody recognizing both the hsOBM and mouse soluble OBM (msOBM) was in on the order of  $10^{-8}$  M with respect to msOBM and in on the order of  $10^{-9}$  M with respect to hsOBM. In addition Further, the dissociation constant of the other regarding H-OBM 13, which was another antibody which recognizes recognizing both antigens shown in the Table 1, i. e. the dissociation constants of H-OBM 13 for each antigen, was the same as that with respect to both antigens were almost identical with those of H-OBM 9, and these since two both antibodies belong to had the same subclass. These findings suggest the, a possibility was suggested that they are were the identical antibodies which recognizes same antibody recognizing the same epitope of each antigen.

<[Example 36]>



**Method for Measuring method of human Human OBM and sOBM by sSandwich ELISA uUsing aAnti-hHuman OBM/sOBM mMonoclonal aAntibodiesy**

A sandwich Sandwich ELISA was constructed using by use of the two types of high affinity monoclonal antibodies obtained in Example 35, i.e., H-OBM 1 and H-OBM 4, respectively as a solid phase antibody and an enzyme- labeled antibody, respectively. Labeling of Maleimide Activated Peroxidase Kit (Pierce Co., Ltd.) was used for labeling the antibody was carried out using a maleimide activated peroxidase kit (Piers Co.). The H-OBM 1 antibody, H-OBM-1, was dissolved in a 0.1 M sodium bicarbonate hydrogen carbonate solution teat a concentration of 10 µg/ml, and 100 µl of the resulting solution was added to each well in of 96-well immunoplates (Nunc company Co., Ltd.). After being allowed The plate was left to stand at 4°C overnight at so 4°C as to immobilize attach the antibody. After the solution in each well was discarded and, 300 µl of 50% Block Ace™ solution BLOCKACE was added to each well, in and the plates. Each well in the plates plate was blocked by allowing left to stand at room temperature for two 2 hours so as to cause blocking. After the blocking, the plates werewas washed with phosphate buffered saline containing 0.1% Ppolysorbate 20 (PBS-P). Human-OBM (hOBM) soluble sOBM and human-soluble- OBM (hsOBM) each were respectively diluted with dissolved in 0.4 M Tris-HCl buffer, (pH 7.4, 7.4) containing 40% Block Ace™ BLOCKACE (Snow Brand Milk Products Co., Ltd.) and 0.1% Ppolysorbate 20 (Wako Pure Chemicals Co-Industries, Ltd.) (the first primary reaction buffer) and diluted so as to prepare test samples with various concentrations. These test samples with different 100 µl of each of test sample, prepared at various concentrations, werewas added to each well in the amount of 100 µl per well, and reacted the plate was left to the antibody, H-OBM-1 immobilized on each well by incubating stand at room temperature for two 2 hours- so as to cause a reaction. After two hours, the plates were Thereafter, the plate was washed with PBS-P. Next, and 100 µl of a solution of POD- labeled H-OBM 4 antibody in diluted with 0.2 M Tris-HCl buffer, HC (pH-7.4, 7.4) containing 25% Block Ace™ BLOCKACE and 0.1% Ppolysorbate 20 (the second secondary reaction buffer) was added to each well, followed by further incubating. The plate was left to stand at room temperature for two 2 hours so as to cause a reaction. The plates were then After the plate was washed with PBS-P and, 100 µl of an enzyme substrate solution (TMB, ScyTek

Co., Ltd.) was added to each well to start enzyme reaction. The enzyme reaction was terminated by so as to develop color in the addition wells, and 100  $\mu$ l of a reaction termination stopping solution (stopping reagent, ScyTek Co., Ltd.) was added to each well so as to stop the enzyme reaction. The absorbance of each well at 450 nm of each well was measured using by use of a microplate reader. The results are shown in Figure Fig. 27.

As a result, it was confirmed revealed that the sandwich ELISA constructed using by use of the two types of high affinity anti-human OBM/sOBM monoclonal antibodies obtained in Example 35, i.e., H-OBM 1 and H-OBM 4 with high affinity for 4, detected human OBM/ and human sOBM prepared in the Example 35, equally recognizes human OBM and human sOBM, and is able to measure a very small amount of human OBM or human sOBM at a quantitative limit of. The measurement sensitivity thereof was about  $1.25 \times 10^{-3}$  to  $2.5 \times 10^{-3}$  pmol/ml (about 50- to 100 pg/ml for human OBM with having a molecular weight of about 40 kDa, about 40- to 80 pg/ml for human sOBM with having a molecular weight of about 32 kDa), and very small amounts of human OBM and human sOBM could be measured by the ELISA. The hybridomas which produce Hybridomas producing these two types of anti-human OBM/sOBM monoclonal antibodies, H-OBM 1 and H-OBM 44, were named H-OBM1 and H-OBM4, respectively. The Further, a hybridoma producing H-OBM 9, the anti-human OBM/sOBM monoclonal antibody (H-OBM 9) which recognizes recognized both mouse OBM and mouse sOBM and also has an osteoclast formation exhibited osteoclastogenesis inhibitory activity, was named H-OBM9. These hybridomas were deposited with the National Institute of Bioscience and Human Technology, of the Agency of Industrial Science and Technology, on November 5, 1993 of the Ministry of International Trade and Industry with Deposit Nos. numbers FERM BP-6264 (H-OBM 1), FERM BP-6265 (H-OBM 4), and FERM BP-6266 (H-OBM 9), on November 5, 1997.

<[Example 37]>

**Measurements of mMouse OBM and mMouse sOBM uUsing aAnti-hHuman OBM/sOBM monoclonal antibody which recognizes mouse Monoclonal Antibody Recognizing Mouse OBM and mMouse sOBM**

~~A sandwich ELISA was constructed~~Sandwich ELISAs using the anti-human OBM/sOBM monoclonal antibody; H-OBM-9, ~~which recognizes~~9 recognizing mouse OBM and mouse sOBM ~~and obtained in Examples 33 and 35 as an a solid -phased antibody-in, and using the Examples 33 and 35, and the~~ anti-mouse OBM/sOBM polyclonal antibody ~~obtained in Example 28 as an enzyme- labeled antibody-obtained in the example 28., were constructed.~~ The mouse Mouse OBM and mouse sOBM were respectively mouse sOBM were diluted stepwise with the first primary reaction buffer to give various concentrations in the same manner as in the Example 35 and then measured sOBM according to the method described in the Example 35, and the mouse OBM and mouse sOBM were detected in the same manner as in Example 36. The results are shown in ~~Figure~~Fig. 28. As a result, it was ~~found~~confirmed that the mouse OBM and mouse sOBM can ~~could~~ be similarly measured using H-OBM-9 ~~which is the detected equally by use of the~~ anti-human OBM/sOBM monoclonal antibody ~~recognizing H-OBM 9 which recognized~~ the mouse OBM and mouse sOBM ~~of the present invention.~~ As shown ~~by in~~ the results of Example 35, ~~this anti-human OBM/sOBM monoclonal~~ the antibody H-OBM 9 ~~has~~ a high dissociation constant relatively with respect to the mouse sOBM; ~~in other words, namely it has a comparatively~~ the antibody had relatively low affinity ~~to for the~~ mouse sOBM. ~~The sensitivity in~~ Thus, the measurement sensitivities of mouse OBM (molecular weight; about 40 kDa) and mouse sOBM (molecular weight; about 32 kDa) ~~by this the above ELISA assay was were~~ about  $25 \times 10^{-3}$  pmol/ml (about 1 ng/ml for mouse OBM ~~and,~~ about 0.8 ng/ml for mouse sOBM).

<[Example 38]>

#### Assay for Osteoclastogenesis- iInhibitory. aActivity of aAnti-OBM/sOBM aAntibody

It is known that an osteoclast-like cells (OCL) ~~are is induced~~derived by co-culture of mouse spleen cells and ST2 cells (mouse bone marrow- derived ~~stromal cells;~~ interstitial cell) (Endocrinology, 125, 1805-1813, 1,805 to 1,813 (1989)). Capability Thus, it was examined whether derivation of the anti- OCL was inhibited by addition of an OBM/ sOBM antibody ~~to inhibit the OCL formation when added to the co-culture system was studied.~~ ~~Because the~~ Since mouse OBM ~~is was~~ expressed in this co-culture system, antibodies used in this Example were H-OBM 9 and rabbit anti-mouse OBM/sOBM polyclonal antibody which recognizesrecognizing mouse OBM ~~and an anti-human.~~ The

OBM/sOBM monoclonal antibody (H-OBM-9) which recognizes both human OBM and mouse OBM antigens were used as the antibodies in this example. Seven hundred microliters per well of each anti-OBM antibody were diluted serially stepwise with  $\alpha$ -MEM containing 10% FCS and 350  $\mu$ l added to a 24 well plate (Nunc Co., Ltd.) in an amount of 700  $\mu$ l/well of, and male mouse splenocytes spleen cells suspended in the above medium ( $2 \times 10^6$  /ml) suspended in the same medium described above were also added to each well in a 24-an amount of 350  $\mu$ l/well plate (Nunc). Next Then, ST2 cells trypsinized and ST2 cells were suspended ( $8 \times 10^4$  cells/ml) in the above-mentioned culture medium containing  $4 \times 10^{-8}$  M  $\gamma$ -vitamin D<sub>3</sub> and  $4 \times 10^{-7}$  M Dexamethazone ( $8 \times 10^4$  cells/ml) were dexamethasone, and the resulting suspension was added to each well in the an amount of 350  $\mu$ l/well, followed by culturing for six. The plate was incubated at 37°C for 6 days at for 37°C culture. After the plates were washed once with PBS once, the cells in each well were fixed with a mixture of 50% ethanol and 50% acetone (50:50) for one hour at room temperature for a minute. The plates were After the plate was air-dried in air, and 500  $\mu$ l of substrate solution was added to each in an amount of 500  $\mu$ l/well according to their accordance with a protocol of the LEUKOCYTE ACID PHOSPHATASE a leukocyte acid phosphatase kit (Sigma Co., Ltd.), followed by incubating and the plate was left to stand at 37°C for 55 minutes at 37°C so as to cause reaction. Only the cells exhibiting the By this reaction, cell showing tartaric acid- resistant acid phosphatase activity (TRAP activity), which is a specific marker for of osteoclasts, were stained by this reaction. The plates were After the plate was washed once with distilled water once and air-dried in air, and the number of TRAP-positive cells was counted. The results are shown in Table 4. As shown in Table 4, a result, it was found that both of the rabbit anti-mouse OBM/sOBM polyclonal antibody and the anti-human OBM/sOBM monoclonal antibody, H-OBM 9, which recognizes mouse OBM9 inhibited derivation of OCL formation in a dose-dependent manner depending on the concentrations of the antibody. These It was found that these antibodies were found to possess had osteoclastogenesis- inhibitory activity like as in the case of an osteoclastogenesis-inhibitory factor, OCIF/OPG, and thus are promising were useful as a therapeutic agent medicament for treating bone metabolism abnormality symptoms.

TABLE 4

Table 4

Amount of antibody (μg/ml)	Number of TRAP positive multinucleates	
	Rabbit anti-mouse OBM/sOBM polyclonal antibody	Mouse anti-human OBM/sOBM monoclonal antibody (H-OBM 9)
0	1155±53	1050±45
10	510±24	650±25
100	10±3	15±4

Amount of Antibody Added (ng/ml)	Number of TRAP Positive Multinucleate Cells	
	Rabbit Anti-Mouse OBM/sOBM Polyclonal Antibody	Mouse Anti-Human OBM/sOBM monoclonal antibody (H-OBM 9)
0	1,155 ± 53	1,050 ± 45
10	510 ± 24	650 ± 25
100	10 ± 3	15 ± 4

(Average ± standard deviation, n = 3)

<[Example 39]>

#### Human osteoclast formation inducing activity of Trx-OBM

#### Osteoclastogenesis Inducing Activity of Trx-OBM

Mononuclear cells were prepared from whole blood collected from the vein of a healthy normal adult by density gradient centrifugation using Histopaque (Sigma Co., Ltd.) according to the protocol with density gradient technique in accordance with an attached protocol. The mononuclear cells were suspended at a cell density concentration of  $1.3 \times 10^6$  cells/ml in  $\alpha$ -MEM containing  $10^{-7}$  M dexamethasone, 200 ng/ml of macrophage colony stimulating factor (The Green Cross Corp/Midori Juji Co., Ltd.), 10% fetal bovine serum, and stepwise concentration (0 to 100 ng/ml) of purified Trx-OBM (0-100 ng/ml) obtained in Example 15. The cell suspension was added to each well in 48-well plates in the amount of 300 μl per well, and the cells were cultured. The plate was incubated at 37°C for three days for culturing cells. After that, the culture broth medium was replaced with the new (identical with above-mentioned culture medium), and the cells were cultured. The plate was incubated at 37°C for four days. The culture medium was removed, and the cells were stained with tartrate-resistant acid phosphatase (TRAP) activity. Cell showing TRAP activity were selectively stained according to the method described in Example 5. The number of stained

multinucleates/multinuclear cells was measured by counted under the microscope observation. The results are shown in Figure Fig. 29. It was confirmed that TRAP-positive multinucleates were induced in As a dose dependent manner by addition of Trx-OBM result, while no TRAP-positive cells showing TRAP activity were hardly detected in the wells to which containing no Trx-OBM, while TRAP positive multinuclear cells appeared in a manner depend on concentration of Trx-OBM when Trx-OBM was not added. Moreover Further, these TRAP-positive multinucleates were found cells showed positive to result for vitronectin receptor which is a marker for of osteoclasts. Furthermore In addition, when similar the cells same culture was carried out conditions were used on ivory dentin slices/fragments placed on each well in a 48-well plate, pit formation was observed on the ivory slices/absorption cavities were formed on the surface of dentin fragments only in the presence of Trx-OBM. Based on these findings, Trx-OBM was formed to have the Thereby, it was revealed that Trx-OBM had activity of inducing human osteoclast to induce formation of human osteoclasts.

<[Example 40]>

#### Inhibition of bone resorbing activity by anti-OBM/sOBM antibody

##### Bone Resorption Inhibitory Activity of Anti-OBM/sOBM Antibody

15-day pregnant ddy mice (Nippon SLC Co., Ltd.), 25  $\mu$ Ci of [ $^{45}$ Ca] -CaCl<sub>2</sub> solution (Amersham Co.) was, Ltd.) were injected subcutaneously injected to ddY mouse (Japan SLC Co.) in the 15th day of pregnancy at a dose of 25  $\mu$ Ci per mouse to label the bone of the fetus, and fetal bones were labeled with  $^{45}$ Ca. Next On the following day, the mouse was sacrificed mice were slaughtered, and their abdomens were opened to obtain remove fetuses from the fetus uteruses. The forefoot of the fetus was drawn and A forelimb was removed from the fetus, the skin and muscle were removed to obtain the take out a long bones. The bone, and a cartilage on the long bone was also removed so as to obtain leave only the shafts diaphysis of the long bones. The shafts of long bones were Each diaphysis was floated one by one in 0.5 ml of culture medium (BGJb medium (GIBCO-BRL company Gibco Co., Ltd.) containing a 0.2% bovine serum albumin (Sigma Co., Ltd.)) in each well in 24 well plates, and cultured for 24 hours at 37°C in the presence of 5% CO<sub>2</sub> for 24 hours. After completion of the pre-cultivation culture, the bones were long bone was transferred into various fresh to a new culture medium (0.5

ml), each containing ~~one of different~~ various bone resorbing factors (vitamin D<sub>3</sub>, prostaglandins E<sub>2</sub>, parathyroid hormone, interleukin 1- $\alpha$ ), and normal rabbit IgG (100  $\mu$ g/ml; as a control); or the rabbit anti-OBM/sOBM polyclonal antibody ~~prepared~~ obtained in Example 28, followed by ~~further cultivation~~ and then cultured for ~~another~~ 72 hours. After completion of the cultivation culture, the long bones ~~were placed~~ in ~~was put into~~ 0.5 ml of ~~an aqueous solution of 5% trichloroacetic acid~~ aqueous solution (Wako Pure Chemicals Co. Industries, Ltd.); and ~~allowed to stand~~ treated at room temperature for ~~more at~~ than least 3 hours ~~so as to decalcify~~ to be decalcified. ~~Five~~ To the conditioned medium and the trichloroacetic acid extract (0.5 ml each), 5 ml of a scintillator (AQUASOL-2, ~~PACKARD~~ Packard Co., Ltd.) was added ~~to the culture broth~~ and the extract of the trichloroacetic acid solution (each 0.5 ml) to measure the radioactivity of <sup>45</sup>Ca, ~~whereby the ratio was measured~~. The proportion of the <sup>45</sup>Ca which ~~was liberated into~~ in the culture broth by solution due to bone resorption was calculated. The results are shown in ~~Figures~~ Figs. 30 to 33. As a result, although the vitamin D<sub>3</sub> (10<sup>-8</sup> M) was found to cause increase the of bone resorbing activity, but the bone resorption caused by the vitamin D<sub>3</sub> was inhibited by addition of the rabbit anti-OBM/sOBM polyclonal antibody suppressed the bone resorption stimulated by vitamin D<sub>3</sub> in a concentration-dependent manner, and the bone resorption was completely ~~inhibiting~~ inhibited by addition of the increased bone resorption antibody at a concentration of 100  $\mu$ g/ml (Figure 30). ~~Prostaglandins~~ Fig. 30). Further, although bone resorption activity was increased in the presence of prostaglandin E<sub>2</sub> (10<sup>-6</sup> M) and/or the parathyroid hormone (100 ng/ml) ~~also increased the bone resorbing activity~~. However, the bone resorption caused by the prostaglandin E<sub>2</sub> or the parathyroid hormone was almost completely inhibited by the addition of 100  $\mu$ g/ml of the rabbit anti-OBM/sOBM polyclonal antibody almost completely inhibited the bone resorption stimulated by ~~prostaglandins E<sub>2</sub> and parathyroid hormone~~ (Figures 100  $\mu$ g/ml) (Figs. 31 and 32). ~~On~~ Meanwhile, ~~the other hand,~~ normal rabbit IgG (100  $\mu$ g/ml), ~~which was used as a~~ positive control, ~~did not affect the bone resorbing activity induced by prostaglandins E<sub>2</sub>~~ and had no effects on the bone resorption by the prostaglandin E<sub>2</sub> and the parathyroid hormone. ~~Bone~~ Further, although bone resorption was also increased by induced by the interleukin 1- $\alpha$  (10 ng/ml), ~~but as well,~~ the bone resorption was inhibited significantly

~~inhibited by the addition of rabbit anti-OBM/sOBM polyclonal antibody (100 µg/ml)~~  
(~~Figure~~Fig. 23). ~~Based on~~From these results, it ~~is~~was ~~clear~~revealed that the antibody of the present invention ~~is~~was ~~excellent as a superior bone resorption inhibitory substance as a bone resorption inhibitor.~~ ~~The results obtained by similar~~As a result of conducting the same experiment using H-OBM 9 which ~~is~~was a mouse anti-human OBM/sOBM antibody, ~~it was~~confirmed that ~~this antibody exhibits an almost~~H-OBM 9 had approximately equivalent bone resorption- inhibitory effect as~~activity to that of the rabbit anti-OBM/sOBM polyclonal antibody.~~

### **Industrial Applicability**

The present invention provides a novel protein ~~that specifically~~which binds to osteoclastogenesis- inhibitory factor (OCIF), a ~~process~~method for ~~preparing the protein~~production thereof, a method for screening~~method for~~ a substance which controls expression of ~~this~~protein using this~~by use of the protein~~, a ~~screening~~method for screening a substance which ~~inhibits or modulates~~the activity of ~~this~~protein, a ~~screening~~method for screening a receptor which binds the protein and ~~transmits the~~an activity of ~~this protein by binding thereto~~thereof, a pharmaceutical composition ~~which contains the~~comprising a substance obtained by ~~these~~said ~~method for screening~~methods, an antibody ~~for~~to the ~~said~~said protein, and an agent for treating bone metabolism abnormality which is formulated using the antibody.

~~Moreover, the present invention provides a DNA encoding~~Furthermore, the present invention provides a DNA which encodes a novel protein (OCIF- binding molecule) which binds to osteoclastogenesis- inhibitory factor (OCIF), a protein ~~which possesses~~having an amino acid sequence encoded by the DNA, a ~~method for preparing the~~method for genetically producing a protein which specifically ~~binding to~~binds to the OCIF ~~using said~~by use of the DNA by a genetic engineering technique, and an agent for treating bone metabolism comprising ~~said~~the protein ~~for treating bone metabolism~~acatastasia. ~~Furthermore, the present invention provides a~~Moreover, methods are provided for ~~screening method for~~ a substance which controls the expression of the OCIF- binding molecule, a ~~screening~~method for screening a substance which binds to the OCIF binding molecule and ~~inhibits or modulates~~the activity of ~~the~~OCIF-



~~binding molecule by binding thereto thereof, a screening method for the screening a~~  
~~receptor which binds OCIF binding molecule and transmits the activity of the OCIF-~~  
~~binding molecule by binding thereto thereof, and a pharmaceutical composition which~~  
~~contains the comprising a substance obtained by the said method for screening methods.~~

Still further, the present invention provides a DNA encoding ~~Also provided is:~~  
DNA, which encodes a novel human protein ~~capable of binding to osteoclastogenesis-~~  
~~inhibitory factor, OCIF-(human-derived OCIF- binding molecule, human OBM) which~~  
~~binds osteoclastogenesis inhibitory factor (OCIF), a protein containing and having an~~  
amino acid sequence encoded by the DNA, a ~~process method for preparing genetically~~  
~~producing~~ a protein ~~having characteristics of which~~ specifically binds OCIF and  
~~exhibiting~~ has a biological activity to support and promote the osteoclast differentiation  
and maturation of osteoclasts by means use of genetic engineering technique the DNA, and  
an agent for treating bone metabolism abnormality using comprising the protein.  
Furthermore, the present invention provides

Also provided are: a screening method for screening a substance which controls  
expression of the OCIF- binding molecule, a screening method for screening a substance  
which binds the OCIF binding molecule and inhibits or modulates the activity of the  
~~OCIF binding molecule by binding thereto thereof, a screening method for the screening a~~  
~~receptor which binds the OCIF binding molecule and transmits the biological activity of~~  
~~the OCIF binding molecule by binding thereto thereof, and~~ a pharmaceutical composition  
~~which contains the comprising a substance obtained by the said method for screening~~  
~~methods, as well as~~ an antibody to the human-derived OCIF- binding protein, and an  
agent for preventing and/or treating bone metabolism abnormality symptoms which is  
formulated using the antibody.

~~In addition~~ Moreover, the present invention provides ~~antibodies~~ an antibody (anti-  
OBM/sOBM antibody) which recognizes both of the following antigens (~~anti-~~  
~~OBM/sOBM antibodies~~), one i.e., a membrane-bound protein- binding molecule (OCIF  
binding molecule; OBM) which specifically binds to an OCIF (~~OCIF binding molecule;~~  
~~OBM~~), and the other a soluble OBM (sOBM) which does not have a lacking membrane  
binding regionsites, a ~~process method for preparing~~ production of the antigen body, a  
method for measuring the OBM and sOBM using these antibodies by use of the antibody,

and an agent for preventing and/or treating bone metabolism abnormality  
~~symptoms which using~~ comprise the antibody as an ~~effective~~ active component ~~ingredient~~.

The ~~proteins and~~ or antibodyies ~~prepar~~ sented by the ~~process of~~ the present invention are useful as ~~medicines and/or~~ medicaments, experimental reagents or diagnostic reagents for research and test purposes.

~~Description of deposited microorganisms~~

(1) ~~Name and address of the depository organization to which microorganism was deposited~~

Reference to Deposited Microorganisms

(1) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry  
1-3,1-3 Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan (postal code (zip: 305)

Date of deposition to the depository organization Deposit:

May 23, 1997

~~The deposition number~~

Deposit Number:

FERM BP-5953

(2) ~~Name and address of the depository organization to which microorganism was deposited~~

(2) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry  
1-3,1-3 Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan (postal code zip: 305)

Date of deposition to the depository organization Deposit:

August 13, 1997

~~The deposition number~~

Deposit Number:

FERM BP-6058

~~(3) — Name and address of the depository organization to which microorganism was deposited~~

(3) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry  
1-3,1-3 Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan(postal code(zip: 305)

~~Date of deposition to the depository organization~~Deposit:

~~November 5, 1997 (Original deposition date)~~

~~The deposition number~~

Deposit Number:

FERM BP-6264

~~(4) — Name and address of the depository organization to which microorganism was deposited~~

(4) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry  
1-3,1-3 Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan(postal code(zip: 305)

~~Date of deposition to the depository organization~~Deposit:

~~November 5, 1997 (Original deposition date)~~

~~The deposition number~~

Deposit Number:

FEPRM BP-6265

~~(5) — Name and address of the depository organization to which microorganism was deposited~~

(5) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry

1-3,1-3 Higashi-1-Chome, Tsukuba-shi, Ibaraki-ken, Japan(postal code(zip: 305)

Date of ~~deposition to the depository organization~~Deposit:

November 5, 1997-(~~Original deposition date~~)

~~The deposition number~~

Deposit Number:

FERM BP-6266